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Elevated Vitamin Levels in Cerebrospinal Fluid in Multiple Sclerosis.* (24097)

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Very few studies have been reported on vitamin content of CSF (cerebrospinal fluid) in health and in disease. Since vitamins supply the prosthetic groups of enzymes, observations of their absence, or inability of absorption or transmission to the proper tissues may be forged into tools to elucidate specific metabolic dysfunctions; vice versa their pathological accumulation may be correlated with hyperfunction(1). Cyanocobalamin and folic acid (PGA) are known to provide the enzymatic apparatus for important steps in the synthesis of nucleic acids. Disturbances in distribution of these vitamins may interfere with the transfer of methyl groups and upset the equilibrium between ethanolamine and choline, or between the respective phosphatides cephalin and lecithin(2). These chemical changes in turn may give rise to demyelination, a histological picture of obscure, but undoubtedly enzymatic etiology(3).

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Part of results were presented at Internat. Congress of Neurological Sciences, Brussels, Aug. 1957.

Methods. The quantities of these vitamins in body fluids, tissues and organs are of such minute order of magnitude that conventional methods of chemical analysis cannot be used, but one has to resort to microbiological assay (4). The analysis of PGA offers particular intricacies, since PGA occurs conjugated to variable extent with peptides of variable molecular weight, requiring enzymatic deconjugation previous to microbioassay; the enzymatic material which must be introduced is itself a source of folic acid which considerably complicates the analysis. We have described (5) a microbioassay, using a thermophilic bacillus which grows rapidly on a simple, purely synthetic medium and which responds equally well to various conjugates as to free folic acid, thus obviating previous enzymatic deconjugation. In the case of Vit. B_{12} preference has hitherto been given to the flagellate *Euglena gracilis* over various strains of *Lactobacillus* used for this purpose, because of its higher sensitivity and specificity(6). However, even *Euglena* shows a wider spectrum than appears desirable, as it responds to certain congeners of Vit. B_{12}, which are ineffective in man and

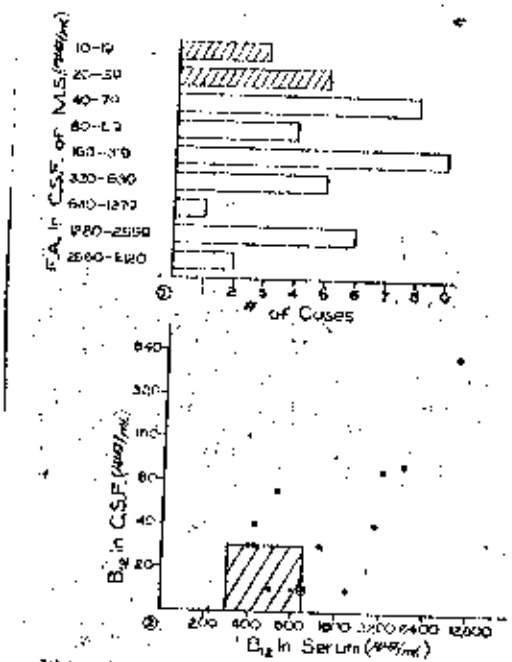

FIG. 1. Folic acid levels in CSF of 43 cases of multiple sclerosis. Normal range 10-30 mμg, hatched staves.

FIG. 2. B_{12} levels in CSF and serum in 20 cases of multiple sclerosis. Normal level in serum 300-1000 μμg/ml. Normal level in CSF 0-30 μμg/ml. Hatched area.

higher animals. We therefore use a test with *Ochromonas malhamensis*, which has been reported to cover those forms of B_{12} to which higher animals respond(7).

Results. We first established the levels of B_{12} and PGA in normal CSF, obtained from a number of non-neurological cases, prior to operative procedures. The normal range of folic acid in CSF is 10-30 mμg/ml; PGA appears to be evenly distributed between serum and CSF.

The figures for B_{12} are 0-30 μμg/ml in CSF as against 300-1000 μμg/ml in blood serum. Vit. B_{12} is known to pass the glomerular membrane without much hindrance, but its rather elevated molecular weight causes considerable difficulty in crossing the blood-brain barrier.

Comparison of vitamin levels in 310 unselected neurological cases shows an unexpectedly high incidence of values above the range observed in normal controls. The patients in our series did not receive vitamin medication. Deviations are found in PGA level of CSF and in B_{12} level of both serum and CSF. Their incidence appears to be concentrated in patients with multiple sclerosis, (MS) comprising a group of 43 cases.

Here PGA was found elevated in CSF in 34 out of 42 cases tested (Fig. 1). The levels of B_{12} in CSF were elevated in 14 out of 41 cases. Amongst these, 9 cases were higher than twice the upper limit of the normal range. The B_{12} values in blood serum of MS patients show a similar incidence of elevated values with 6 out of 20 cases above 1000 μμg/ml and 7 additional cases at 1000 μμg/ml, which is considered the upper limit of the normal range for this vitamin. The values for B_{12} in CSF and serum show fair correlation (Fig. 2).

Discussion. In pernicious anemia administration of folic acid initially benefits the patient clinically, but it seems to mobilize all available stores of B_{12}, primarily in liver and possibly in bone marrow; thus depletion of the organism of B_{12} after a few weeks gives rise to severe neurological symptoms (subacute combined degeneration of the cord). No analogy to this functional antagonism between the 2 vitamins exists in MS. No correlation was found between high PGA and low B_{12} values. In fact 4 cases among the highest B_{12} values in serum and CSF are ranking high among the folic acid results.

Because of the intermittent nature of MS, a single specimen can give us only a cross-sectional view of a surmisedly unstable situation. In view of the possibility that deviations in level of serum and CSF constituents may be detectable only during acute episodes, the observed incidence of elevated values is as high as may be expected and gains considerable significance. High vitamin values in CSF may be involved in the etiology of MS, or may act merely as indicators of changes in permeability of the blood-brain barrier. That this permeability is subject to variations was observed in a collateral study, where very high PGA and B_{12}-levels in CSF of neurological patients dropped abruptly within 5 min. during electric shock therapy, indicating a temporary breakdown of the blood-brain barrier. These observations support the concept that

vitamin B_{12} is involved in various pathological cerebral manifestations(8), which are ultimately based on enzymatic disturbances. Decreases and increases(9) of various hydrolases in degenerating nervous tissue have been reviewed by Cavanagh and Thompson (3). Some of these enzymes (*e.g.* the glycosidase synthesizing nucleosides) as well as transmethylase are interrelated with B_{12} and FGA metabolism.

Summary. The folic acid and Vit. B_{12} content of CSF and B_{12} in serum was studied in more than 300 neurological cases. The FGA levels in CSF in a number of cases were elevated; high values were scattered among a variety of neurological conditions, but were high in four-fifths of cases with MS. The titre of B_{12} in CSF and serum of MS patients was elevated in one-third and reached the upper limit of the normal range in another third of the cases. We assume that increased levels of these vitamins in CSF are not merely an indication of increased permeability of the blood-brain barrier, but of etiological significance for MS.

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Folate and Vitamin B_{12} Metabolism in Weanling Rats Given a Maize Diet

I. Folate and Vitamin B_{12} Concentrations in Liver and Serum

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The association of megaloblastic anemia with protein malnutrition in infants subsisting on a maize diet is well recognized.[1–4] The validity of standard tests of folate and vitamin B_{12} nutrition in this syndrome is thus of considerable importance, particularly in view of recent evidence that in protein-malnourished infants some of the common indices of folate and vitamin B_{12} nutrition may not accurately reflect body stores of these vitamins.[5–8] The significance of these indices can best be determined by comparison with the results of direct measurement of folate and vitamin B_{12} concentrations in the liver, the major organ of storage for these vitamins. In view of the impracticability of obtaining sufficient liver tissue from infants, a study of rats given a maize diet was undertaken to determine the relationship between serum and liver concentrations of folate and vitamin B_{12} during protein depletion and subsequent refeeding.

MATERIAL AND METHODS

Subjects

Newborn rats, not inbred, of Wistar stock were used for study. Rats were weaned at four weeks, and then allocated randomly to four different dietary regimens. Each group comprised ten animals. All animals were kept in separate cages designed to prevent coprophagy.

Group 1. Rats received the stock diet and were sacrificed after six, eight, ten and twelve weeks.

Group 2. Rats received the maize diet for six weeks. After this period some were sacrificed whereas others were then given the stock diet, either unsupplemented or supplemented with vitamin B_{12} (100 μg. in a single weekly intramuscular injection) or pteroylglutamic acid (PGA, 1 mg. intramuscularly twice a week). The rats that had been refed were then sacrificed at intervals of two weeks.

Group 3. Rats received the maize diet with PGA (1 mg. intramuscularly twice a week). These rats were sacrificed after six weeks.

Group 4. Rats received the maize diet, together with vitamin B_{12} (100 μg. weekly in a single intramuscular injection). These rats were sacrificed after six weeks.

Diets

Stock Diet. The commercial diet was known to be adequate for normal growth and nutrition of the rat. The vitamin B_{12} concentration of the diet, assayed by the method of Bannerjee and Chatterjea[9] using papain digestion, averaged 27 μmg. per gm. wet weight and the folate activity, assayed with Lactobacillus casei according to Toepfer et al.[10] using pancrease digestion, averaged 350 μmg. per gm. wet weight.

Maize Diet. Commercial maize meal flour (180 gm.) was cooked in 1 L. water, with salt and sugar added. The vitamin B_{12} content of cooked samples of the mixture averaged 0.16 μmg. per gm. wet weight and the L. casei folate activity averaged 28 μmg. per gm. wet weight.

Both diets were given *ad libitum*.

Procedure

At the desired time rats were anesthetized lightly and the abdominal wall opened. Blood for assay of serum L. casei folate activity and vitamin B_{12} concentration was collected to exsanguination from the point of bifurcation of the abdominal aorta. The

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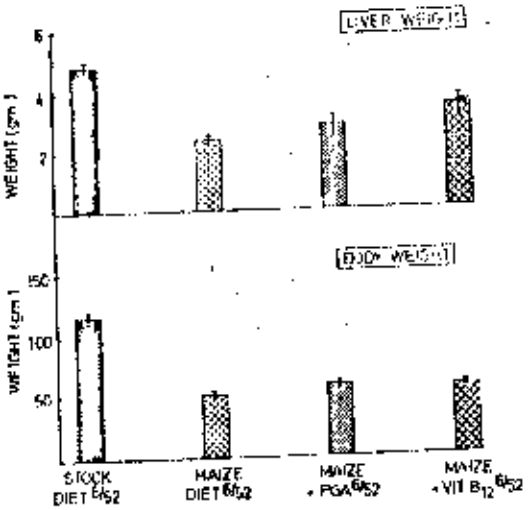

FIG. 1. Liver and body weights of rats given different diets for six weeks. The fraction of 52 refers to the number of weeks of the dietary regimen. $\bar{x}$ = mean ± standard error.

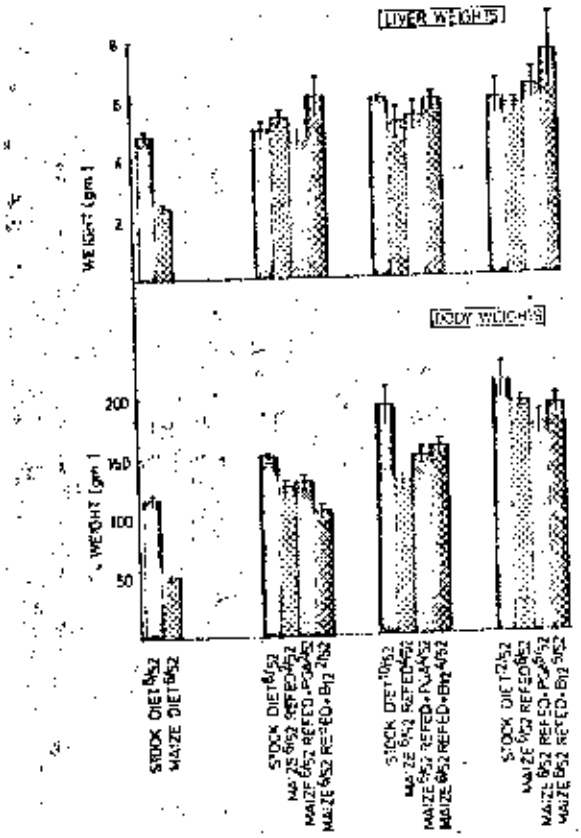

FIG. 2. Effect of refeeding on liver and body weights of rats. The fractions of 52 refer to the number of weeks of the dietary regimen. $\bar{x}$ = mean ± standard error.

exsanguinated livers were removed, weighed and stored at −20°C. until assayed. The blood samples were centrifuged and the serum removed and stored at −20°C. until assayed. Ascorbate was not added to the serum and liver samples to be stored, as all samples were assayed within four weeks of collection. Serum folate activity was not assayed in rats given supplements of PGA, or serum vitamin B_{12} concentration in rats given supplements of vitamin B_{12}.

Assay of Serum Folate and Vitamin B_{12}

Serum folate activity was assayed with L. casei by the method of Herbert[11] and Waters and Mullin,[12] and with Streptococcus faecalis according to Chinard et al.[13] Serum vitamin B_{12} was assayed with Lactobacillus leichmanii, as described previously.[14]

Assay of Liver Concentration of Folate and Vitamin B_{12}

Folate. Weighed portions of liver were homogenized in ascorbic acid phosphate buffer, incubated and filtered as described by Bennett et al.[15] Suitable dilutions of the filtrate were assayed with L. casei using Difco® Bacto folic acid casei medium 0922-15, with S. faecalis using the medium of Toepfer et al.,[16] and with Pediococcus cerevisiae using Difco Bacto C.F. assay medium 0156-15. The study was carried out prior to publication of a report by Bird et al.[17] who showed that P. cerevisiae activities of liver samples which have undergone autolysis may not be a true reflection of the original P. cerevisiae activity in the sample. Therefore the results of assays with P. cerevisiae are not included herein.

Vitamin B_{12}. Weighed portions of liver were homogenized, mixed with sodium cyanide, boiled and centrifuged, as described by Booth and Spray.[17] Suitable dilutions of the clear supernatant were assayed with L. leichmanii using Difco Bacto B_{12} assay medium USP 0457-15.

All samples for microbiologic assay were assayed in triplicate in at least two separate batches. Results were expressed per gram of liver.

RESULTS

Body and Liver Weights (Fig. 1 and 2)

Weanling rats given the maize diet failed to gain weight (Table 1) and after six weeks their mean body weight was 41 per cent that of control rats. On refeeding the stock diet (Fig. 2) body weight increased rapidly, but remained

slightly less than that of control rats even after six weeks. The mean liver weight of the rats fed maize was 50 per cent that of control rats. On refeeding, gain in liver weight was rapid and after two weeks the mean value was comparable to that of control rats.

Liver and Serum Folate Activity (Fig. 3 and 4)

The patterns of change in liver folate activity of rats fed maize were generally similar when measured with L. casei or S. faecalis. The mean liver folate concentrations in weanling rats given a maize diet for six weeks was of the order of 30 per cent that of control rats (Fig. 3). However, this difference was not reflected by the serum L. casei folate levels; the mean value obtained for the rats fed maize was slightly higher than that for control rats. When the maize diet was supplemented with PGA the mean liver folate concentration was greater than that of control rats. Supplementation with vitamin B_{12} prevented a significant reduction in liver folate concentration, but the serum L. casei level was only 52 per cent that of control rats.

In rats given the maize diet for six weeks and then refed with the stock diet (Fig. 4) the liver folate concentration after two weeks had increased, the mean values being slightly higher in rats receiving vitamin B_{12} supplements. After four and six weeks the mean concentrations in rats refed the unsupplemented stock diet were comparable to those of control rats; animals receiving PGA showed the highest concentrations, whereas those receiving vitamin B_{12} showed concentrations higher than those given the unsupplemented stock diet.

Liver and Serum Concentrations of Vitamin B_{12} (Fig. 5 and 6)

In weanling rats given a maize diet for six weeks the mean liver vitamin B_{12} concentration was 25 per cent that of control rats (Fig. 5). This depletion in vitamin B_{12} stores was reflected by a fall in the serum vitamin B_{12} level to 20 per cent that of control rats. Supplementation of the maize diet with PGA did not prevent the development of vitamin B_{12} depletion, but supplementation with vitamin B_{12} resulted in a marked increase in the liver vitamin B_{12} concentration, compared to control rats.

TABLE 1
Failure of Growth of Rats After Six Weeks on Maize Diet

Stage	Mean Body Weight ± S.E. (gm.)	Mean Liver Weight ± S.E. (gm.)
Weanling	49.22 ± 1.44	1.80 ± 0.03
After 6 wk. on maize diet	50.76 ± 1.63	2.37 ± 0.11
After 6 wk. on stock diet	115.10 ± 5.36	4.77 ± 0.25

When rats given a maize diet for six weeks were subsequently refed the stock diet, the mean serum vitamin B_{12} concentration increased rapidly, and after two weeks it was greater than that of control rats (Fig. 6). The liver concentration of vitamin B_{12} was restored less rapidly, but was comparable to that of control rats after four weeks. Supplementation with vitamin B_{12}, but not PGA, accelerated the rate of increase in liver concentration of vitamin B_{12}.

COMMENTS

Weanling rats given a maize diet failed to grow and multiple nutritional deficiencies were probably produced. The diet was deficient in protein and amino acids, as well as folate and vitamin B_{12}, as shown by assay of these vitamins in the maize meal that was fed. After six weeks of the maize diet the mean body weight, liver weight, and liver concentrations of folate and vitamin B_{12} were considerably less than those of control rats. This severe folate and vitamin B_{12} depletion in rats fed maize can be directly attributed to the inadequate dietary intake of these vitamins. The folate depletion is in accord with other reports of depleted folate concentrations in liver of rats given a diet deficient in these vitamins (Davis,[18] Kelly and Davis,[19] Grossowicz et al.[20]).

In rats fed maize with depleted folate and vitamin B_{12} stores, the serum vitamin B_{12} concentration was reduced to 20 per cent that of

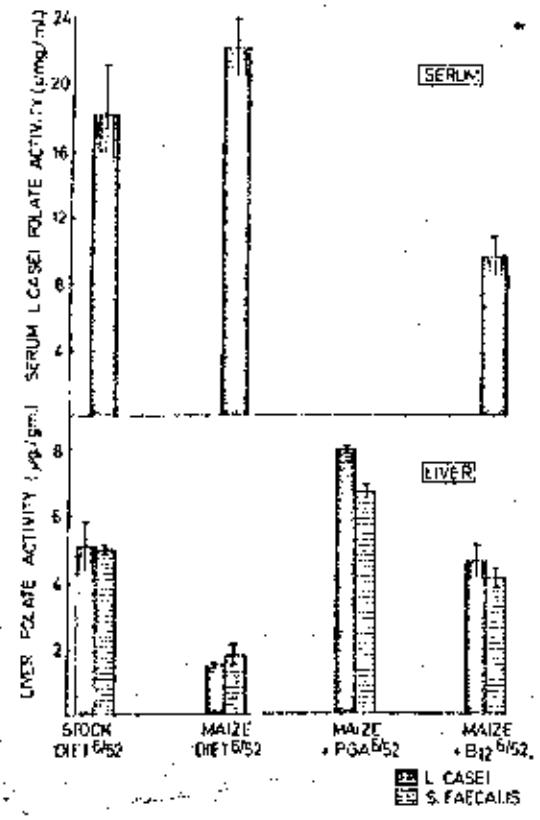

FIG. 3. Serum and liver folate activity in weanling rats given different diets for six weeks. The fraction of 6/52 refers to the number of weeks of the dietary regimen. ‡ = mean ± standard error.

control rats. Thus depletion of body vitamin B_{12} stores was reflected by a fall in the serum level of vitamin B_{12}. This was not, however, the case with the serum L. casei folate level. Despite a marked reduction in liver folate activity, the serum L. casei levels were slightly higher than those of control rats. This failure of the serum L. casei level to reflect body folate deficiency when there was associated severe vitamin B_{12} deficiency is in accordance with observations on folate metabolism in vitamin B_{12} depletion in man. In pernicious anemia there is often increased L. casei folate activity in the serum.[18] Herbert and Zalusky[21] found slow disappearance of L. casei activity from the serum after PGA was given intravenously to patients with vitamin B_{12} deficiency; when large doses of vitamin B_{12} were given daily to these patients, clearance of L. casei serum activity became rapid. These observations led the latter workers to postulate a "pile-up" of L. casei-active material in vitamin B_{12} deficiency. The metabolic interrelationships of folate and vitamin B_{12} are complex and as yet incompletely understood. Nevertheless, a single metabolic reaction in which both function (*viz.*, the conversion of homocysteine to methionine) has been well established.[21] In this reaction N^5-methyl-tetrahydrofolic acid supplies a methyl group to homocysteine to convert it to methylhomocysteine (methionine), which process requires vitamin B_{12} acting as a coenzyme. When vitamin B_{12} deficiency occurs, N^5-methyl-tetrahydrofolic acid (for which

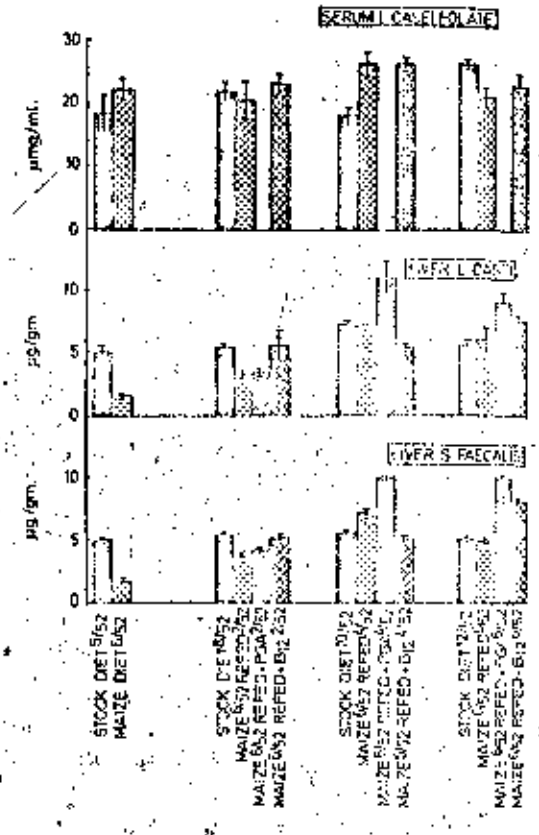

FIG. 4. Effect of refeeding with stock diet, with and without vitamin supplementation, on serum and liver folate activity of rats given a maize diet for six weeks. The fractions of 6/52 refer to the number of weeks of the dietary regimen. ‡ = mean ± standard error.

only L. casei is active) cannot be utilized adequately and accumulates in the serum.

The results of the serum and liver folate estimations in weanling rats given maize diets supplemented with large doses of vitamin B_{12} are of interest. The vitamin B_{12} supplement enabled these rats to maintain their liver folate concentrations at a normal level, only slightly less than that of control rats. This took place despite the very low folate content of the diet. Grossowicz et al.[30] found that in rats deprived of folate but not of vitamin B_{12} there was a similar, although slightly smaller, decrease in serum folate activity. It appears possible that when vitamin B_{12} is available in liberal quantity to the rat subsisting on a folate-poor diet, folate stores can be mobilized from other sites to the liver. Evidence for this concept is afforded by the findings of Grossowicz et al. who showed that in rats given a folate-deficient diet, liver stores of folate decreased at a significantly slower rate than stores at other sites.[30] That folate nutrition was not, however, completely adequate in the present study of rats given maize diets supplemented with vitamin B_{12} is shown by the 50 per cent fall in serum L. casei levels. This confirms that the serum level is probably a highly sensitive index of dietary folate intake, and underlines the previous observation that in experimental folate deficiency in man a fall in the serum L. casei level was the first index of folate depletion after commencing a folate-free diet.[28]

The results of the studies carried out during refeeding may have some application in the

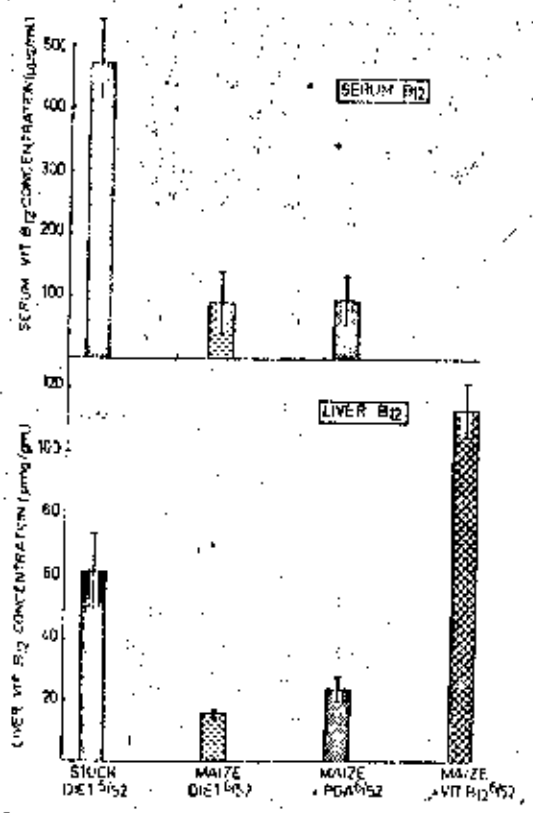

FIG. 5. Serum and liver concentrations of vitamin B_{12} in weanling rats given different diets for six weeks. The fraction of 52 refers to the number of weeks of the dietary regimen. ± = mean ± standard error.

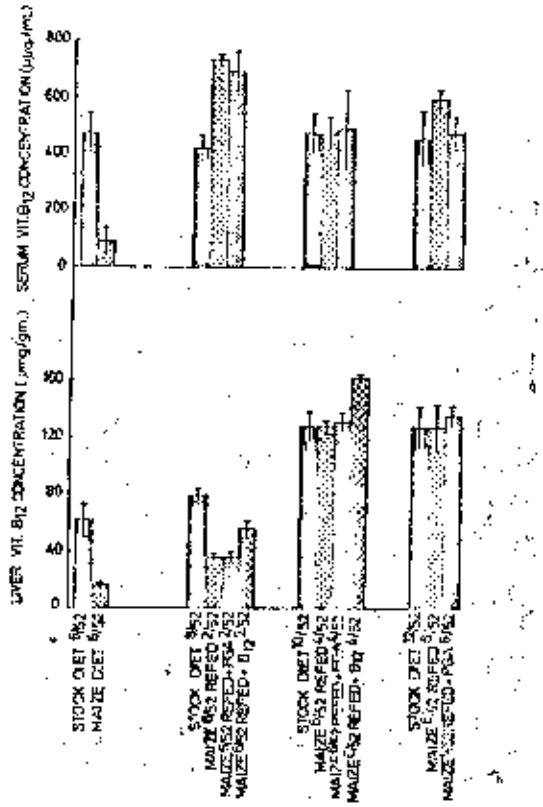

FIG. 6. Effect of refeeding with stock diet, with and without vitamin supplementation, on serum and liver vitamin B_{12} concentrations of rats given a maize diet for six weeks. The fractions of 52 refer to the number of weeks of the dietary regimen. ± = mean ± standard error.

clinical management of protein-malnourished infants. On refeeding, the folate and vitamin B_{12} concentrations in the liver increased rapidly and the rate of increase was not accelerated by supplementation with these vitamins. It appears questionable whether protein-malnourished infants should be supplemented with PGA and/or vitamin B_{12} once refeeding with an adequate diet has been established.

SUMMARY

In weanling rats given a maize diet for six weeks, severe depletion of liver folate and vitamin B_{12} concentrations occurred. The depletion of liver vitamin B_{12} concentration was reflected by a fall in serum levels of vitamin B_{12}, but the serum Lactobacillus casei folate activity did not reflect the depleted folate stores. These observations are in accordance with the concept of a "pile-up" of L. casei-active material, in the form of N^5-methyltetrahydrofolic acid, in vitamin B_{12} deficiency.

When weanling rats fed maize were given supplements of pteroylglutamic acid (PGA) liver folate concentration was maintained, but vitamin B_{12} deficiency was not prevented. Supplementation with vitamin B_{12} prevented depletion of both vitamin B_{12} and folate in the liver, but the serum L. casei folate activity decreased markedly.

Refeeding a stock diet to rats previously subsisting on a maize diet resulted in a rapid increase in the liver concentration of folate and vitamin B_{12}. Supplementation with PGA and vitamin B_{12} did not significantly accelerate the repletion of the liver stores in refed rats.

ACKNOWLEDGMENT

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Failure of L-Glutamic Acid to increase Absorption of Vitamin B_{12} by Patients with Pernicious Anaemia

Heatherote and Mooney[1] reported results from which they concluded that vitamin B_{12} is more efficacious therapeutically in patients with pernicious anaemia when given by mouth with five times its weight of glutamic acid than when given alone. This claim was based on the haematological responses of two patients. The observation suggested that glutamic acid may enhance the absorption of vitamin B_{12} from the gut in pernicious anaemia. If confirmed, this could have an important bearing on theories concerning the mechanism of the absorption of vitamin B_{12} from the gut. We therefore decided to see whether addition of glutamic acid to test doses of cyanocobalamin labelled with cobalt-58 influenced the absorption by patients with pernicious anaemia, using the total body counter described by Warner and Oliver[2].

Table 1. Failure of L-Glutamic Acid to Increase the Absorption of ^{58}Co-Vitamin B_{12} by Patients with Pernicious Anaemia

Patient No.	Afebrile	Percentage of dose of 0·5 μg ^{58}Co-cyanocobalamin retained when given: With 50 mg hog intrinsic factor concentrate	With 5 μg glutamic acid	With 50 mg glutamic acid
1	0	72	0	–
2	4	73	8	–
3	10	57	17	10

L-Glutamic acid was dissolved in hot water and, after cooling, suitable quantities of the solution were added to 20 ml. of solution containing the test dose of 0·5 μg cyanocobalamin labelled with cobalt-58, shortly before it was given to the patients. The results (Table 1) show that neither 5 μg nor 50 mg glutamic acid increased absorption, although when an intrinsic factor preparation was given with the test dose, each patient absorbed much more. If glutamic acid does in fact enhance the therapeutic effect of vitamin B_{12} given by mouth, our results suggest that it must influence some process other than absorption.

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Vitamin B_{12}-binding Proteins in Normal and Leukemic Human Leukocytes and Sera

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Abstract: STENMAN, U.-H., SIMONS, K. & GRÄSBECK, R. Vitamin B_{12}-binding Proteins in Normal and Leukemic Human Leukocytes and Sera. *Scand. J. clin. Lab. Invest.* 21, 202-210, 1968. The vitamin B_{12}-binding proteins in sera and leukocytes of healthy subjects and patients with chronic myelogenous leukemia (CML) were separated and partially purified by column chromatography as their cyanocobalamin complexes. The molecular weight of transcobalamin I and leukocyte B_{12}-binding protein was 119,000, and that of transcobalamin II was 32,000 as determined by gel filtration. The electrophoretic mobilities were α_1 for transcobalamin I, α_1 for the leukocyte B_{12}-binding protein, and α_2-β for transcobalamin II. After treatment with neuraminidase or chromatographic purification, the mobilities of both transcobalamin I and the leukocyte B_{12}-binding protein became β. Transcobalamin I and the leukocyte B_{12}-binding protein gave a reaction of immunologic identity in immunodiffusion. The fact that leukocytes can produce B_{12}-binding protein in vitro and the similarities between leukocyte B_{12}-binding protein and transcobalamin I strongly suggest that CML transcobalamin I is derived from leukocytes.

Key-words: Blood proteins; gel filtration; leukemia; leukocytes; macromolecular systems; transcobalamins; vitamin B_{12}

Vitamin B_{12} (B_{12})* in serum is transported by two trace proteins—transcobalamin I (TC I) and transcobalamin II (TC II) (12). TC I has an α_1 electrophoretic mobility (20, 23) and carries the 'endogenous' B_{12} in serum (23). TC II normally bears little B_{12} and takes up most of the vitamin added to serum in vitro or in vivo. Its electrophoretic mobility is α_2-β (11, 20).

Leukocytes contain considerable amounts of a B_{12}-binding protein (LB) with properties similar to the R-proteins without intrinsic factor activity in gastric juice, saliva, and bile (26). Earlier studies showed that such a protein is synthesized by leukocytes in vitro, and that its molecular weight determined by gel filtration is about 115,000 (29).

The B_{12}-binding capacity in serum from patients with chronic myelogenous leukemia (CML) is increased (1, 22). The increase correlates fairly well with the peripheral white cell count (20). The vitamin is bound by a protein which has the same electrophoretic mobility as TC I (21). It is not clear whether this binding protein (CML TC I) is identical with normal TC I, and how these binding proteins are related to the leukocyte binding protein.

*Non-standard abbreviations:
CML=Chronic myelogenous leukemia, TC I=transcobalamin I, TC II=transcobalamin II, LB=leukocyte B_{12}-binding protein, B_{12}=vitamin B_{12}.

The aim of this study was to investigate the relationship between TC I, TC II, and I.F in normal subjects and in patients with CML, and to determine the molecular weight, electrophoretic mobility, and chromatographic and immunologic properties of these proteins.

MATERIAL AND METHODS

Serum or plasma from normal subjects or patients with CML in relapse was used fresh or stored at −20° C until used.

Leukocytes were isolated from heparinized blood by the method of Killman (17). One part of a 6 per cent dextran solution (Dextran 250, Pharmacia, Sweden) was added to nine parts of blood and the erythrocytes were allowed to sediment. The buffy coat was separated and washed thrice in saline. This leukocyte preparation was frozen and lysed by sonic oscillation, as described (29).

^{57}Co-cyanocobalamin (Radiochemical Centre, England), specific activity of 10-300 μCi/μg, was used to label the binding proteins. One ng ^{57}Co-B_{12} was added per ml of normal serum; enough B_{12} to saturate the binding capacity was added to CML serum and lysed leukocytes. The excess was removed by dialysis.

The binding proteins were isolated by chromatography on DEAE-Sephadex (Pharmacia, Sweden), CM-cellulose (Schleicher & Schuell, Germany), and CM-Sephadex (Pharmacia, Sweden). Stepwise elution was used throughout. For DEAE-Sephadex 5 mM phosphate pH 7.1 (equilibration buffer), followed by 0.07 M NaCl and 0.17 M NaCl. For CM-cellulose the steps were 0.02 M acetate pH 5.4, 0.04 M phosphate pH 7.8, and 0.2 M phosphate pH 7.8, and for CM-Sephadex 5 mM phosphate pH 5.5 and 0.2 M phosphate pH 7.8.

'Molecular weights' (or more correctly molecular sizes) were determined by gel filtration on a Sephadex G-150 (Pharmacia, Sweden) column (1.5 × 87 cm) in 0.1 M phosphate buffer, pH 7.4. At this ionic strength TC II does not form complexes (14). The equation of Determan & Michel (2) was used:

$$\log M = \log M_0 - (6.062 - 5.893d)\, V_e/V_0.$$

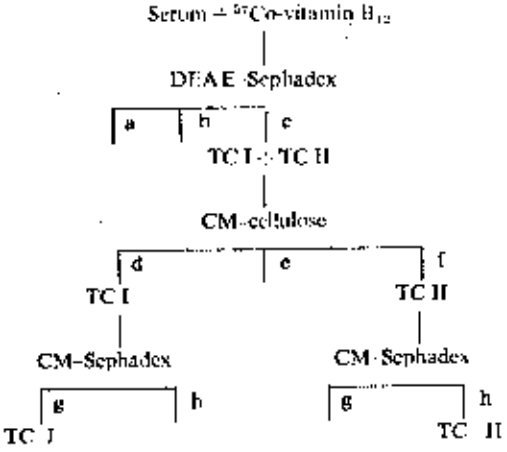
The gel constant M_0 for the column was determined with bovine albumin (Armour fraction V, Armour, USA), 1.03 was used as the value for d (2). The void volume was determined with Blue Dextran 2000 (Pharmacia, Sweden), which was mixed with the sample under study.

Zone electrophoresis in Pevikon, a copolymer of polyvinyl chloride and polyvinyl acetate (Stockholms Superfosfats Fabriks AB, Sweden), was used to determine the electrophoretic mobility of the B_{12}-binding proteins. The buffer was 0.1 M Veronal, pH 8.6. Four 0.5 ml samples were applied on a block measuring 14 × 42 cm, thickness 0.7 cm. A serum sample was included and albumin was stained with bromphenol blue. With the usual conditions (6.1 V/cm for 17 hrs) albumin migrated about 20 cm. After the run the block was cut into segments 1 cm wide and the radioactivity of each segment was recorded in a well-type scintillation counter. The protein content of the eluates of the segments was determined by the Lowry procedure (19).

Rabbit antisera against human leukocytes (8), gastric juice (27), and saliva (30) were used for immunoelectrophoresis, by the micromethod of Scheidegger (25), and for immunodiffusion by a modification of the Ouchterlony technique (13). The radioactive precipitin lines were demonstrated by autoradiography.

The purified preparations of CML TC I and the binding proteins of normal and CML leukocytes were treated with neuraminidase (Behringwerke, Germany). One ml of the neuraminidase solution (500 i.u.) and 2 ml of a 0.05 M acetate buffer containing 0.9 per cent NaCl and 0.1 per cent $CaCl_2$ were added to 1 ml of the sample. This mixture was dialysed for 16 hours at 37° C against the same buffer to remove free sialic acid. Controls were dialysed without neuraminidase.

Scheme 1. Chromatographic separation of TC I and TC II. The letters indicate buffers: a) 5 mM phosphate pH 7.0, b) 0.07 M NaCl, c) 0.17 M NaCl, d) 0.02 M acetate pH 5.4, e) 0.04 M phosphate pH 7.8, f) 0.2 M phosphate pH 7.8, g) 5 mM phosphate pH 5.5, h) 0.2 M phosphate pH 7.8.



RESULTS

Chromatography

The chromatographic procedure is illustrated in Scheme 1. Radioactive B_{12} was added to normal serum. The mixture was dialysed overnight against 5 mM phosphate buffer pH 7, and chromatographed on DEAE-Sephadex. All the radioactivity was eluted with the last buffer, 0.17 M NaCl (Fig. 1 A). The radioactive fractions were pooled, ultrafiltered, and dialysed against the equilibration buffer of the next step. When the pool was chromatographed on CM-cellulose two radioactive components emerged (Fig. 1 B). The first was eluted with the equilibration buffer and contained about 10 per cent of the radioactivity. The rest of the radioactivity was eluted with the last buffer. The first component corresponds to TC I and the second to TC II (12). The TC II peak was further purified on CM-Sephadex. Most of the radioactivity was eluted with the second buffer (Fig. 1 C). This component was concentrated and used as TC II. The TC I peak from the CM-cellulose step came through with the equilibration buffer in CM-Sephadex chromatography.

CML serum was purified in the same way. Its elution pattern in DEAE-Sephadex chromatography was like that of normal serum (Fig. 1 D). All the radioactivity was eluted with the last buffer 0.17 M NaCl. When this component was pooled and chromatographed on CM-cellulose it was split into two (Fig. 1 E), as was normal serum. The first peak, corresponding to TC I, contained a much larger proportion of the radioactivity eluted than the first peak from normal serum. The values ranged from 50 to almost 100 per cent, depending on the amount of TC I in the different sera. This component was chromatographed on CM-Sephadex and nearly all the activity came through with the equilibration buffer (Fig. 1 F). This component was used as CML TC I. The TC II peak from CM-cellulose was eluted with the second buffer in CM-Sephadex, as was TC II from normal serum.

The binding protein from normal and CML leukocytes was purified in the same way as the serum proteins. On DEAE-Sephadex all the radioactivity was eluted with 0.17 M NaCl (Fig. 1 G). On CM-cellulose this component behaved like TC I and practically all the radioactivity came through with the equilibration buffer (Fig. 1 H). This fraction was further chromatographed on CM-Sephadex. The pattern was similar to that of TC I. Most of the activity came directly through the column (Fig. 1 I), but some radioactivity was usually eluted with 0.2 M phosphate. The first peak was concentrated and used as the leukocyte B_{12}-binding protein (LB). Much of the radioactivity became dialysable during ultrafiltration. The second component, which was eluted with 0.2 M phosphate, lost even more activity during ultrafiltration and was not studied further. There were no differences between the elution patterns of LB from normal and CML leukocytes (Fig. 1 J-L).

The binding proteins prepared in this way

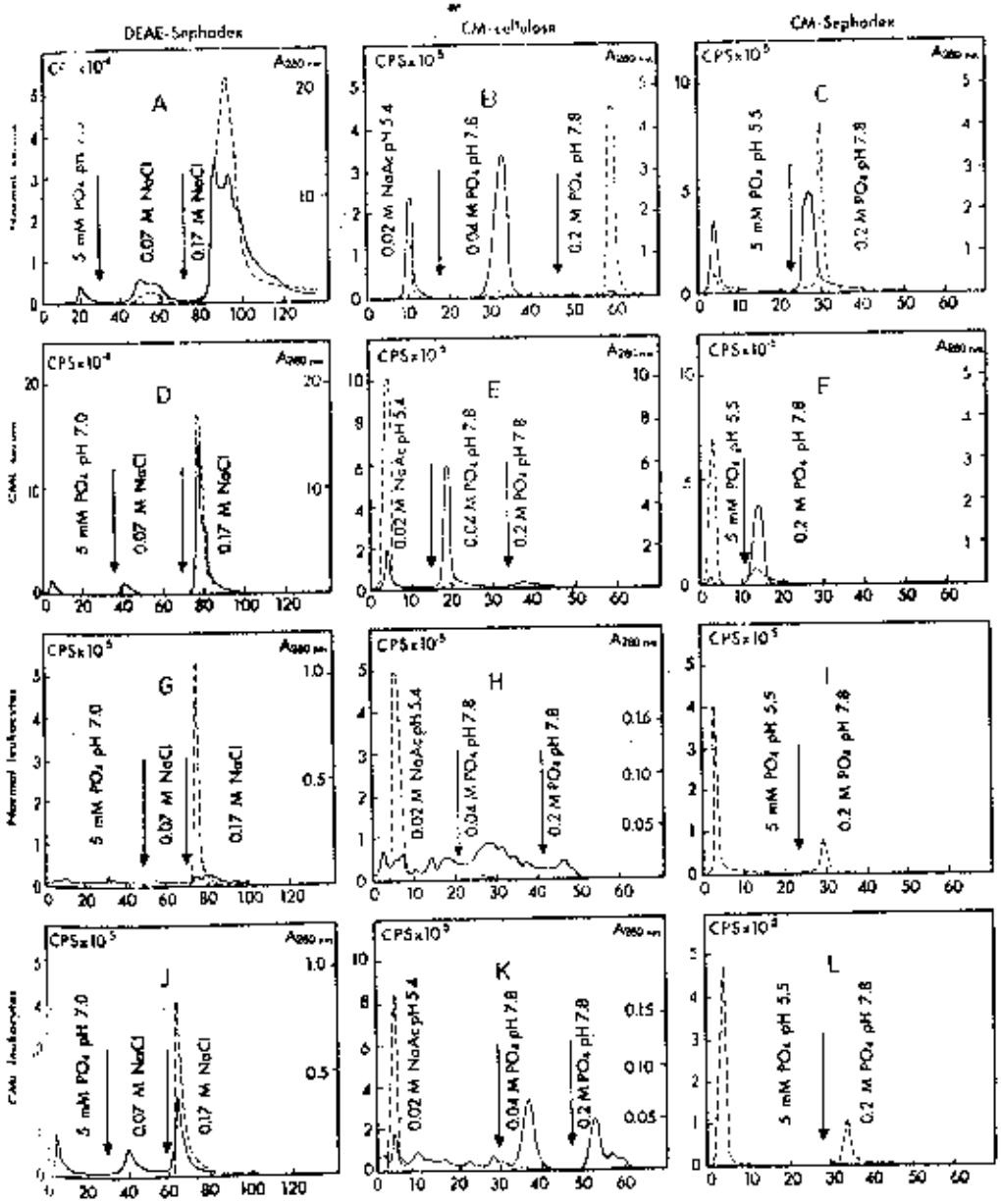


Fig. 1. Three-step purification of normal and leukemic sera and leukolysate by DEAE-Sephadex, CM-cellulose, and CM-Sephadex chromatography. Absorbance at 280 nm– – ——, radioactivity - - - - - - -. Abscissa fraction number, fraction volume 10 ml. The protein concentration was too low to be measured in I and L.

A–C: Purification of normal transcobalamin II.
D–F: Purification of transcobalamin in chronic myelogenous leukemia serum.
G–I: Purification of normal leukocyte B_{12}-binding protein.
J–L: Purification of leukocyte B_{12}-binding protein in chronic myelogenous leukemia.

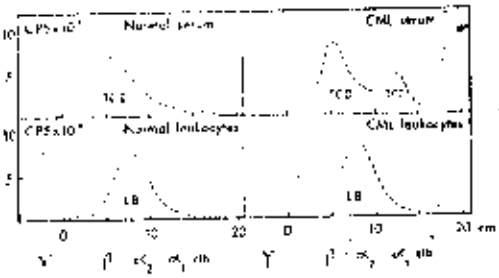


Fig. 2. Pevikon electrophoresis of normal and leukemic leukolysate and sera. Application point at 0 cm, anode to the right.

were free from contaminating B_{12}-binding proteins as evidenced by symmetrical radioactive peaks in gel filtration.

Electrophoresis

Fig. 2 shows the patterns obtained with normal and CML serum in Pevikon electrophoresis. TC I was in the α_1 region and TC II in the α_2-β region. Free vitamin, when present, remained at the starting point or moved slightly towards the cathode. The binding protein of lysed but otherwise untreated leukocytes formed a symmetrical peak in the α_2 region (Fig. 2). It moved somewhat faster than TC II but clearly more slowly than TC I. No difference in mobility was detected between the binding proteins from normal and CML leukocytes (Fig. 2).

Binding proteins that had been purified by the chromatographic steps described had considerably slower mobilities than those in the starting material. Purified CML TC I moved in the β region as compared with α_1 for unpurified TC I. Purified LB also had β-mobility; originally it was α_2. Purified TC II was also slower than that in unfractionated material, the change being from α_2-β to β. The β-mobility of purified CML TC I and LB was also seen in immunoelectrophoresis, as shown in Fig. 3.

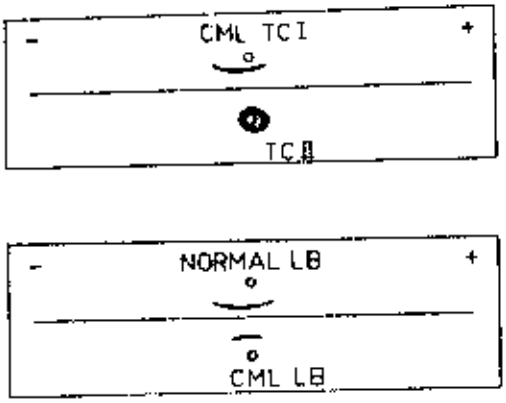


Fig. 3. Immunoelectrophoresis of the B_{12}-binding proteins with anti-leukocyte serum. CML TC I, normal and CML LB formed radioactive precipitin lines. Precipitation around the application point was observed with TC II. The application points and the antiserum troughs have been drawn on the autoradiogram.

Immunoelectrophoresis

CML TC I and the binding proteins from normal and CML leukocytes formed radioactive precipitin lines with anti-leukocyte, anti-gastric juice, and anti-saliva immune sera. The position of the arcs indicated mobility for both CML TC I and the leukocyte binding protein. Too little TC I was obtained from normal serum to be used for the immunological studies. TC II gave no radioactive lines with the antisera used, but there was some nonspecific precipitation around the application point (Fig. 3).

Immunodiffusion

The purified binding proteins were studied in double diffusion experiments in agar gel against the antisera used for immunoelectrophoresis. Only CML TC I and LB gave visible precipitin lines. The lines of these binding proteins fused with each other (Fig. 4). TC II did not give a precipitin line with the antisera used, neither did it interfere with the arcs of the other binding proteins.

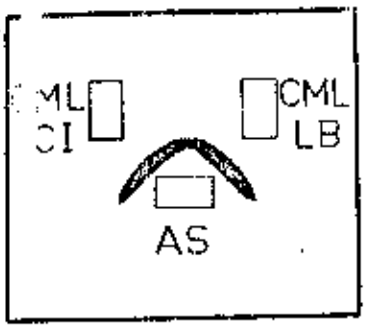


Fig. 4. Immunodiffusion of CML, TC I, and LB against anti-leukocyte serum (AS). The precipitin lines fused indicating antigenic identity. The wells have been drawn on the autoradiogram.

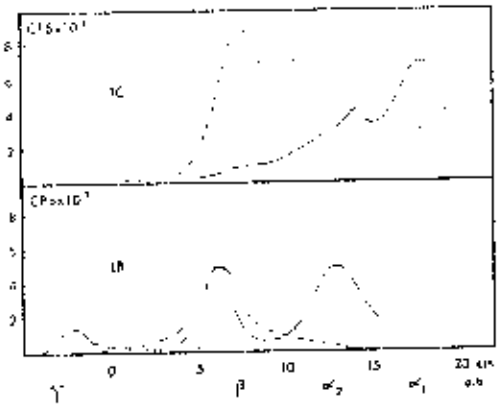


Fig. 5. Pevikon electrophoresis. CML, TC I, and LB treated with neuraminidase - - - - - - - -, controls incubated without neuraminidase ——— ·—·—, Application point at 0 cm, anode to the right. Free vitamin B_{12} moved in the γ-globulin region.

Gel filtration

Purified binding proteins, sera, and lysed leukocytes saturated with radioactive B_{12} were studied by gel filtration in Sephadex G-150. Purified and unpurified binding proteins gave similar results. Values for V_e/V_o and the corresponding molecular weights are given in Table I. Normal and CML TC I and the LB's had the same V_e/V_o value indicating a similar molecular weight of about 119,000. TC II was considerably smaller, about 32,000. These values are close to those obtained by Hom and Oleser: ~121,000 and 38,000 (16).

Neuraminidase treatment

LB and TC I were each incubated with sialidase. LB was purified only with CM-Sephadex, and TC I was prepared from CML serum by a quick two-step purification on DEAE-Sephadex and CM-Sephadex. Most of this TC I preparation had normal α_1 mobility, but an additional slower component with α_2 mobility was seen (Fig. 5). Only one radioactive component with α_2 mobility was seen in the LB preparation (cf. Fig. 2). The mobilities in Pevikon of the neuraminidase-treated binding proteins were compared with those of con-

Table I. *Molecular weights determined by gel filtration*

	V_e/V_o			
	mean	range	Molecular weight	No. of observations
TC I	1.43	1.41-1.45	119,000	4
LB	1.43	1.43-1.44	119,000	3
TC II	2.06	2.04-2.06	32,000	4
Bovine Albumin (standard)	1.70	1.69-1.71	(67,000)	2

trols incubated without neuraminidase. The neuraminidase treatment changed the mobility of CML TC I to β, and that of LB also changed, to β. There was still, however, a small difference in mobility between TC I and LB after the neuraminidase treatment. In the LB control, which was incubated at 37° C for 16 hours, 30 per cent of the α_2 component underwent the same change in mobility as seen in the sample treated with neuraminidase (Fig. 5).

DISCUSSION

The purification system used, i.e. DEAE-Sephadex, CM-cellulose, and CM-Sephadex chromatography, was chosen to separate the B_{12}-binding proteins from each other. The final preparations contained contaminating proteins but, as judged by gel filtration, the binding proteins did not contaminate each other.

Chromatographic purification caused a change in the electrophoretic mobilities of the B_{12}-binding proteins. Prior to purification CML TC I had α_1 mobility and LB α_2. After the three chromatographic steps these proteins had approximately the same mobility, β. TC II also changed its mobility, from α_2-β to β. The change in the mobilities of TC I and LB could also be effected by treatment with neuraminidase in vitro. It is thus possible that sialic acid is split off during the fractionation procedure. The glycoprotein fraction of serum is known to contain neuraminidase activity (31).

Another difficulty noted by us previously (29) and by other investigators (4) was the dissociation of bound B_{12} from the binding proteins during the later stages of purification. This is probably due to denaturation of the B_{12}-binding protein in very dilute solutions. When isolated from a 40 liter pool of gastric juice the intrinsic factor-B_{12} complex was much more stable than when isolated from smaller amounts of starting material (9).

Because of these difficulties (see also Horn, 14) it is clear that many criteria for identity or non-identity must be used when comparing the different B_{12}-binding proteins. CML, TC I, and LB (CML and normal) have identical elution volumes in gel filtration and the antisera available at present indicate that their antigenic properties are also identical. Furthermore, after chromatographic fractionation and neuraminidase treatment these proteins have similar electrophoretic mobilities at pH 8.6. It is therefore likely that the granulocytes are the source of serum TC I in CML, as suggested previously (see Glass, 5). The difference in mobility between TC I in serum and LB of leukocytes in vitro seems to be due to different sialic acid content. In vivo sialic acid may be added to the LB molecule when it is released from the leukocytes (cf. Eylar, 3).

The R proteins, including TC I and LB, seem to occur in most body fluids. Immunologic studies have shown that these proteins have common antigenic determinants (26), and TC I, LB, and the gastric R-protein (10) have similar molecular weights of about 120,000. However, they have different mobilities in electrophoresis (26), possibly due to differences in sialic acid content. Studies with isoelectric focusing have revealed heterogeneity of otherwise homogeneous intrinsic factor preparations. This heterogeneity is at least partly due to differences in sialic acid content (6). The gastric and salivary R-proteins are also heterogeneous in isoelectric focusing (7). The function of TC I and the R-proteins is not known. B_{12} bound to TC I injected intravenously has a relatively long half-life in the circulation, 9-10 days (15).

The human R-proteins enhance B_{12} uptake in rat liver homogenates (28), but it is doubtful whether this phenomenon has any physiological significance.

TC II has been found only in serum. It has a relatively small molecular weight, about 32,000 (cf. 16). B_{12} bound to TC II injected intravenously has a half-life of only about 1.5 hours (15). Several in vivo and in vitro studies indicate a transport function for this protein (4, 12, 15, 24).

There thus exist at least three classes of human extracellular B_{12}-binding proteins, for

...sic f... or, which serves as a transport pro... ...n for ...12 in the gastrointestinal tract, TC I, which ...seems to transport B_{12} in the blood ... the t... ...ies, and the R-proteins, including ...rum T... I and the intracellular I.B, for which ... function is yet known.

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Metabolites of Vitamin B_{12} in Bile after Parenteral Administration of the Vitamin

SEVERAL investigations have shown that, in animals and in man[1], the major proportion of the total radioactivity excreted after an injection of radioactive vitamin B_{12} is excreted in the bile, and that in man at least two-thirds of the biliary radioactivity is re-absorbed from the intestine. This indicates that vitamin B_{12} is subject to an entero-hepatic circulation similar to other substances in bile.

Radioactive bile was collected via a bile duct cannula from rats injected with radioactive vitamin B_{12}. Vitamin B_{12} and radioactive cobalt-containing metabolites of B_{12} were extracted from the bile. The acidified bile (pH 2–3) was denatured with 95 per cent ethanol, and any bound B_{12} or B_{12} analogues freed by boiling in water for 15 min. The supernatant, after evaporation to a small volume, was subjected to repeated extractions with chloroform and then the water-phase (pH 2–3), saturated with ammonium sulphate, was extracted with secondary butanol. The secondary butanol phase, after adding ethyl ether, was extracted with water. Having thereby removed most of the pigment and impurities, compounds containing cobalt were adsorbed on activated charcoal, washed with 5 per cent phenol, and then eluted with hot 65 per cent acetone. The eluate was evaporated to a small volume to which some non-radioactive concentrated vitamin B_{12} solution was added for a control spot on the electrophoretogram and chromatogram. Throughout the extraction, the pH was kept at 2–3 and any cobalamines were maintained in the cyano form by the addition of 0·01 per cent sodium cyanide. Two-dimensional separation of the metabolites was attempted by electrophoresis at a potential of 8 V./cm. for 16 hr. on No. 1 Whatman filter paper in a buffer of 0·5 *N* acetic acid and 0·01 per cent sodium cyanide; followed by descending paper chromatography at right angles to the direction of electrophoresis in a system of water-saturated secondary butanol with 1 per cent acetic acid (w/v) and 0·01 per cent sodium cyanide. The resulting radioactive spots on the chromatogram were resolved by making autoradiographs using X-ray film. The radioactivity in the chromatogram spots was counted in a scintillation counter after elution with water.

Fig. 1 shows the results obtained from a sample of rat bile. The control spot contained only non-radioactive vitamin B_{12}, detected by its colour on the chromatogram, and is represented diagrammatically. The relative amounts of radioactivity contained in

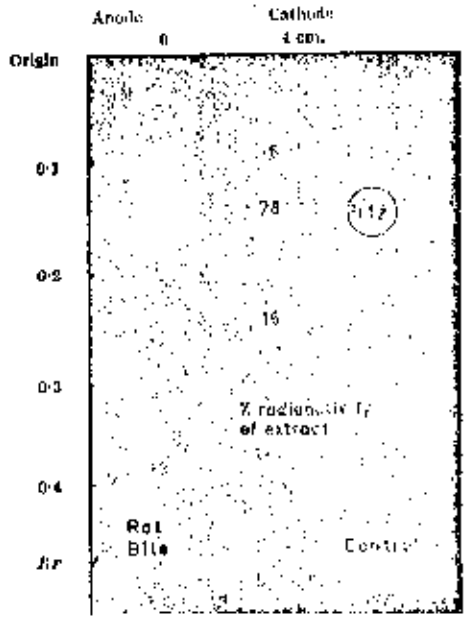

Fig. 1. Autoradiograph of a sample of rat bile following the injection of cobalt-58 vitamin B_{12}

each spot on the chromatogram is shown. A similar result was obtained from a different sample of rat bile.

Comparison with the work of others[1] indicates that the metabolites of vitamin B_{12} here demonstrated in bile are analogues of vitamin B_{12}, and like B_{12} have zero electrophoretic mobility. Free cobalt as cobalt chloride did not migrate in any of the runs shown on the chromatogram. The rat bile contained at least two analogues of vitamin B_{12}. The major portion, approximately three-fourths, of the radioactivity was present as vitamin B_{12}, the remaining fourth being in the B_{12} analogues.

It has been shown by others (see ref. 3) that cyanocobalamine is more readily absorbed in man than the other cobalamines and structural analogues of B_{12}, absorption of the latter being dependent on a strict chemical specificity of the B_{12} benzimidazole group[4]. It seems likely, therefore, that the metabolic products of B_{12} will not be absorbed, or will be poorly re-absorbed once they are excreted in the bile. For this reason, the presence of vitamin B_{12} analogues in bile offers a possible explanation of the appreciable amount of radioactivity sometimes found in the faeces of normal human subjects in the faecal excretion test of B_{12} absorption[5]. Similarly, it offers an explanation of the finding[3] that re-absorption by the enterohepatic circulation of the radioactivity in human bile after parenteral cobalt-56 vitamin B_{12} was incomplete, being approximately two-thirds of the total radioactivity present.

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Experimental Anemias in Animals Resulting from Folic Acid and Vitamin B_{12} Deficiencies

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I. Introduction

Interest in experimental anemias in animals has been stimulated by their possible relation to pernicious anemia in humans. The observations that pernicious anemia was related to atrophy of the gastric mucosa led to many fruitless attempts to produce the equivalent of this human degenerative disease by gastrectomy in a number of experimental animals (Bussabarger *et al.*, 1939; Petri and Jensenius, 1941; Petri *et al.*, 1944). The finding of experimental counterparts to pernicious anemia did not result from a directed search, but rather from nutritional studies proceeding concurrently with several experimental and domesticated animals.

Although the metabolic defects and nutritional deficiencies in pernicious anemia are now well known, there is no nutritional deficiency in any experimental animal that is exactly the same as pernicious anemia. The complete hematological response in this disease to either folic acid or to vitamin B_{12} cannot be duplicated in any experimental animal.

H. Monkeys

A. Folic Acid Deficiency

The rhesus monkey is very susceptible to folic acid deficiency. Since its requirement for ascorbic acid resembles that of man, this species is especially useful for studying the role of ascorbic acid in the etiology of macrocytic anemia. Wills and Billimoria (1932) and Day *et al.* (1935) were the first to produce experimentally and to differentiate clearly what is now known as folic acid deficiency or vitamin M deficiency in the monkey. However, Day (1944) points out in his excellent review that "this syndrome or certain manifestations of it have been rediscovered and described every few years since 1919."

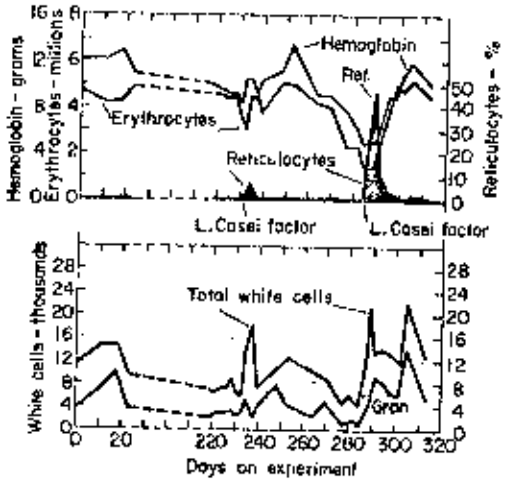

FIG. 1. Typical responses of the cytopenic monkey to natural pteroyltriglutamic acid (*Lactobacillus casei* factor). In each of the two experiments on this monkey the total dose of vitamin was approximately 3 mg, given intramuscularly. Courtesy of Day *et al.* (1945).

The rhesus monkey develops folic acid deficiency on a folic acid-deficient diet without the use of an intestinal antiseptic or folic acid antagonist. Day and his co-workers used a diet based on vitamin-free casein, polished rice, vitamins, and orange juice. On this diet an anemia develops in which there is a progressive decrease in all types of peripheral blood cells (Day, 1944; Totter *et al.*, 1944; Day *et al.*, 1945; Langston *et al.*, 1938). This anemia may be fatal within 30–80 days (Day *et al.*, 1935). Erythrocytes may decrease from a normal value of 5 million to

1 or 2 million. A marked leukopenia is the most characteristic aspect of folic acid deficiency. Total white cell counts may decrease from normal levels of 10,000–20,000 to 2000–3000. There is a marked granulocytosis which is similar to that observed in folic acid-deficient rats. Some animals may develop a marked leukopenia which is fatal without exhibiting any severe anemia (Day, 1944). The bone marrow is hyperplastic and megaloblastic (Wills and Stewart, 1935). The administration of folic acid produces a reticulocyte response as high as 40% and a rapid increase in total white cells to a level that may exceed the normal number, and a gradual increase in hemoglobin and red cell count (Fig. 1), (Day *et al.*, 1945). On the casein and rice basal diet employed by Day (1944), which furnished about 20 μg of folic acid per day, the minimum daily protective dose of folic acid is about 100 μg (Day and Totter, 1948). The minimum amount required to produce a moderate reticulocyte response (7%) is 100–200 μg (Wilson *et al.*, 1946).

B. Megaloblastic Anemia Due to Scurvy

The requirements for folic acid by the rhesus monkey are modified by ascorbic acid deficiency. The relationship between ascorbic acid and folic acid was originally observed in the etiology of megaloblastic anemia of infancy (Zuelzer and Ogden, 1946; May *et al.*, 1950a). May and co-workers (1950a) attempted to reproduce the condition of megaloblastic anemia of infancy in monkeys by feeding milk diets used for human feeding. These diets which consisted of boiled cow milk were found to contain 2.6 μg of folic acid per liter by *Streptococcus faecalis* assay. More recent analysis of cow milk in which ascorbic acid was used to prevent destruction shows approximately 80 μg of folic acid per liter in cow milk and 50 μg per liter in autoclaved milk (Naiman and Oski, 1964). This would give an intake in May's experiments (May *et al.*, 1950a) of 20–30 μg per day. On these milk diets, monkeys did not develop any anemia when they were supplemented with ascorbic acid. When ascorbic acid was omitted scurvy developed at about 85 days and megaloblastic anemia developed 2–3 weeks later. A megaloblastic bone marrow developed, and the hemoglobin dropped to about 5% in 10–30 days. A leukopenia also developed. The administration of folic acid alone without ascorbic acid rapidly eliminated the megaloblastosis. Doses as small as 100 μg per day intramuscularly and 200 μg per day orally were found to be effective in the absence of ascorbic acid (May *et al.*, 1952a). Ascorbic acid alone is effective in relieving the megaloblastosis (May *et al.*, 1952a), although in some cases this return may be gradual (May *et al.*, 1950a). Vitamin B_{12} is ineffective in either preventing or curing the megaloblastosis in monkeys (May *et al.*, 1952a), although vitamin

B_{12} is sometimes effective in curing megaloblastic anemia in infants (May *et al.*, 1950a).

The role of ascorbic acid in affecting the appearance of anemia is not yet clear. It has been observed that leucovorin (5-formyltetrahydrofolic acid) is more effective in relieving megaloblastosis than folic acid; 7.5 μg of leucovorin per day was found to be much more effective than 50 μg of folic acid and somewhat less effective than 750 μg of folic acid (May *et al.*, 1950b, 1953). Ascorbic acid has been reported to increase the *in vitro* conversion of folic acid to citrovorum factor by rat liver slices (Nichol and Welch, 1950) and to increase the urinary excretion of citrovorum factor following ingestion of folic acid. The conversion of folic acid to citrovorum factor or leucovorin (5-formyltetrahydrofolic acid) requires the preliminary reduction of folic acid to tetrahydrofolic acid. Although it can be visualized that ascorbic acid could facilitate this reduction, there is little evidence to show that ascorbic acid is required for or will facilitate the formation of tetrahydrofolic acid by dihydrofolic acid reductase in which TPNH is the hydrogen donor (Blakley and McDougall, 1961; Huennekens, 1963). Ascorbic acid may also function in increasing the microbiologically assayable citrovorum factor by increasing the stability of the tetrahydrofolate derivatives during assay (Bakerman, 1961; Bird *et al.*, 1965). Tetrahydrofolic acid is active for *Pediococcus cerevisiae* only when reoxidation by molecular oxygen is inhibited by ascorbic acid. Thus in the presence of sufficient ascorbic acid tetrahydrofolate would appear as citrovorum factor by assay with *P. cerevisiae* (Bakerman, 1961; Bird *et al.*, 1965).

On the basis of recent information on the enzymatic reduction of folic acid and the formylation of tetrahydrofolate, there is no evidence to support the conclusion that ascorbic acid would directly facilitate the formation of 5-formyltetrahydrofolic acid or any other formyl derivative *in vivo*. This is supported by the fact that the effect of ascorbic acid in increasing *in vitro* formation of citrovorum factor in liver slices is nonspecific and can be duplicated by glucoascorbic acid (Nichol and Welch, 1950). This compound was ineffective as a replacement for ascorbic acid in curing megaloblastic anemia in scorbutic monkeys (May *et al.*, 1952a).

Studies on the content of vitamin B_{12} and folic acid in the liver showed that the total folic acid content of the liver (after conjugase treatment), which is normally 1.0 μg/gm, had decreased to approximately 0.2 μg/gm, in livers of monkeys with megaloblastic marrows (May *et al.*, 1952a). This reduced folic acid content was not related solely to a reduced ascorbate content of the liver, since the liver ascorbate decreased rapidly from a normal value of 120 μg/gm to a value of 12 μg/gm in the first 30 days on the deficient diet while the marked drop in liver folate occurred

at about 100 days when the liver ascorbate was 4 μg/gm. The sharp drop in liver folate to 0.2 μg/gm which occurred at 100 days, shortly after development of scurvy, coincided with the development of megaloblastosis (May *et al.*, 1953). Treatment of scorbutic megaloblastic animals with ascorbic acid caused a rise in liver folate levels to 0.6 μg/gm and development of a normoblastic marrow (May *et al.*, 1952a). It might be noted that the normal liver folate values of 1 μg/gm of fresh liver are much lower than those reported for rat liver. Bird *et al.* (1965) found 10–20 μg/gm in autoclaved rat liver after treatment with conjugase.

Ascorbic acid deficiency does not cause anemia by decreasing intestinal synthesis of folic acid since the folic acid content of the feces is not affected. The intestinal absorption of orally administered folic acid or folic acid conjugate (May *et al.*, 1953) is also unaffected by ascorbic acid. May and his co-workers believe that the appearance of megaloblastosis following development of low liver folate values is caused by nonspecific stress factors occurring in scurvy rather than by any specific chemical action of ascorbate on folic acid metabolism (May *et al.*, 1953). This is supported by the observation that monkeys on adequate diets which develop respiratory infection may develop megaloblastic marrows which are associated with low liver folate values (May *et al.*, 1952b). In animals with severe respiratory infection, the livers of animals with megaloblastic anemia averaged 0.16 μg of folate per gram while in those with normal marrows the livers averaged 0.6 μg of folic acid per gram. Normal monkeys fed the same diets for long periods had liver folate levels of 1.1 μg/gm. The possibility of inflammation as a causative agent is suggested by the find-

TABLE I
EFFECT OF VARIOUS STRESS CONDITIONS ON THE VITAMIN CONTENT OF LIVER OF MONKEYS AND THE DEVELOPMENT OF ANEMIA[a]

Stress condition	Total animals	Ascorbic acid (μ/gm)	B_{12} (μ/gm)	Total folic acid (μ/gm)
Controls, adequate ascorbic acid	5	129	1.0	1.10
Turpentine abscess, adequate ascorbic acid				
Megaloblastic	3	151	0.8	0.30
Scorbutic				
Megaloblastic	7	8	0.6	0.14
Normoblastic, supplementary folic acid	1	4	0.6	1.43
Infection, adequate ascorbic acid				
Megaloblastic	5	103	0.5	0.16
Normoblastic	6	—	0.5	0.62

[a] Data courtesy of May *et al.* (1952b).

ing that a megaloblastic marrow also developed following turpentine injections which produced sterile abscesses (May *et al.*, 1952b). After 3 or 4 injections the marrow became megaloblastic in about 50 days. At this time the liver ascorbate levels were normal (*ca* 150 μg/gm) but the liver folate values dropped to 0.3 μg/gm, which is comparable with the liver folate values of scorbutic monkeys with megaloblastic anemia. These data show that infection and inflammation can reduce liver folate levels and thus induce a megaloblastic anemia. Whether this inflammatory response which occurs in scurvy is comparable with that occurring in turpentine abscess is impossible to say. It does demonstrate that a nutritional deficiency of folic acid may be the result of a secondary effect produced by another etiological agent as well as by a direct effect. The effects of these different stress conditions, such as scurvy, infection, and turpentine abscesses, on the folic acid and vitamin B_{12} content of liver and the development of anemia are shown in Table I (May *et al.*, 1952b).

C. Vitamin B_{12} Deficiency

A deficiency of vitamin B_{12} in the monkey does not appear to produce an anemia similar to pernicious anemia. In the experiments of May *et al.* (1952a) milk diets were used which furnished about 1 μg of B_{12} per day. The administration of vitamin B_{12} did not prevent or give any consistent hematological response in animals which developed megaloblastosis following scurvy. No information was given on serum B_{12} levels in these experiments. Since milk contains a small amount of B_{12}, it may be expected that no vitamin B_{12} deficiency would appear. The use of an all-vegetable protein is required to produce markedly deficient vitamin B_{12} diets. Wilson and Pitney (1955) found that monkeys maintained on a synthetic diet supplemented with 1 μg of vitamin B_{12} per day experienced a drop in vitamin B_{12} serum levels to 25–50 picograms (pg) per milliliter. Under conditions of optimum nutrition on stock diets the serum levels varied from 50 to 230 pg with an average of 120. Administration of either folic acid or ascorbic acid to megaloblastic scorbutic monkeys caused 2- to 4-fold increase in B_{12} serum levels. Administration of 10 μg of vitamin B_{12} per day to monkeys with serum levels of 50 pg caused 10-fold increases in B_{12} serum level, but no mention was made of any hematological abnormality on the basal diet or of any change following administration of vitamin B_{12}. Oxnard (1964, 1966) has reported that rhesus monkeys recently captured have vitamin B_{12} serum levels of 120–330 pg/ml. This progressively decreases during captivity when they are kept on an all-vegetable diet and reach levels of 20–80 pg/ml after 24 months. Hematological examinations revealed no anemia or macrocytosis. No marrow studies were reported.

In pernicious anemia vitamin B_{12} serum levels of less than 80 pg/ml are associated with anemia. It is thus apparent that the monkey does not develop anemia at serum B_{12} levels which are associated with marked anemia in the human.

III. Swine

A. Folic Acid Deficiency

Folic acid deficiencies in pigs have not been observed with purified diets without the use of intestinal antiseptics or folic acid antagonists.

Blood changes have been produced in adult pigs by feeding purified diets with casein as the protein source and which contained sulfonamides. In some of these cases (Cartwright *et al.*, 1946; Cunha *et al.*, 1948) a normocytic anemia developed which seemed to be due to a vitamin B_{12} deficiency, since the administration of purified liver extract produced a marked reticulocyte peak. In subsequent experiments with this same type of diet, Cartwright and Wintrobe (1949) obtained partial responses to liver extract and more marked responses to folic acid. Reduction of the level of crude casein from 20 to 10% made the anemia more severe.

The further addition of a folic acid antagonist, x-methylfolic acid (Franklin *et al.*, 1947), to the synthetic diet containing a sulfonamide produced a severe macrocytic anemia which responded only partially in some animals to purified liver extract and responded markedly to folic acid (Heinle *et al.*, 1948, 1949; Cartwright *et al.*, 1948, 1949, 1950). The hematological manifestations observed with sulfonamide plus the antagonist include (a) a severe anemia with the hematocrit dropping to 20%—this anemia initially was normocytic with numerous macrocytes and a comparable number of microcytes; the macrocytosis continued to develop even in the animals receiving liver extract; (b) leukopenia with a greater proportional reduction of polymorphonuclear cells than mononuclear cells; (c) mild thrombocytopenia; (d) a megaloblastic hyperplastic bone marrow (Cartwright *et al.*, 1948, 1949). The blood and bone marrow were rapidly restored to normal by injection of 10–20 mg of folic acid or the corresponding tri- and heptaglutamates. When 1 unit of liver extract (1 μg vitamin B_{12}) was given, the anemia was not prevented or delayed. When 150 units of liver extract or 150 μg of vitamin B_{12} was used curatively a partial hematological response was obtained, although less than that given by folic acid. Thymine, which produces a hematological response in pernicious anemia, was inactive. Heinle *et al.* (1949) found that in a similar diet containing sulfonamide and an antagonist small doses of folic acid would give an initial response which became smaller with subsequent doses, and that these pigs, which failed to respond to small

doses of folic acid, would respond to liver extract. It is thus obvious that this diet which was acutely deficient in folic acid because of the antagonist was also marginally deficient in vitamin B_{12}.

B. Vitamin B_{12} Deficiency

A markedly vitamin B_{12}-deficient diet requires the use of a vegetable protein in place of casein. Neumann *et al.* (1948) fed a synthetic milk diet to pigs, using isolated soybean protein, and obtained growth increases of approximately 50% by the injection of liver extract or vitamin B_{12} (Johnson and Neumann, 1949). Collaborative hematological studies of these animals (Neumann *et al.*, 1950) showed no differences in numbers of red and white cells. Cartwright *et al.* (1951), however, found that when pigs were kept for over 100 days anemia developed in some animals on a vitamin B_{12}-deficient diet. Out of 40 vitamin B_{12}-deficient pigs, 13 showed no anemia, 16 a mild anemia, and 11 developed a moderate anemia which was normocytic. No significant leukopenia was observed in B_{12} deficiency, but in some animals there was a moderately severe neutropenia. The hypersegmentation of the polymorphic neutrophils which is seen in pernicious anemia (Chanarin *et al.*, 1965) was not observed in swine. Bone marrow examination revealed a slight increase in the proportion of erythroid cells, but no megaloblasts such as those observed in folic acid-deficient pigs or in pernicious anemia patients were found. Although no severe and consistent anemia developed, the animals appeared to be severely deficient in vitamin B_{12} as judged by the reduced growth rate and the marked growth response produced by vitamin B_{12} administration. The hematological disturbances were not increased by administration of desiccated thyroid, iodinated casein, or gluconsorbic acid. Administration of vitamin B_{12} in most cases produced a reticulocyte response which varied from 5 to 25%, with one extreme case as high as 53%. In some cases a reticulocyte response was obtained even when no anemia was present. There was also no correlation between height of the reticulocyte peak and the severity of anemia or the degree of red cell response following therapy. This is in contrast to pernicious anemia where the reticulocyte response to therapy is proportional to the severity of the anemia. It is thus obvious that a vitamin B_{12} deficiency in pigs does not produce a hematological picture similar to that found in pernicious anemia.

C. Combined Vitamin B_{12} and Folic Acid Deficiency

Detailed studies of combined deficiencies of folic acid and vitamin B_{12} have also been made. Johnson *et al.* (1950) used diets containing isolated soy protein and either x-methylfolic acid as an antagonist or sulfathalidine to inhibit bacterial synthesis of folic acid. The first type of diet

produced a severe combined deficiency state which gave a reticulocyte response to either folic acid or vitamin B_{12}. However, the deficiency was so acute with the antagonist that it was necessary to remove the antagonist at 3 weeks, at which time therapy with either folic acid or B_{12} was begun.

Cartwright *et al.* (1952) studied a double deficiency using a diet containing isolated soy protein, succinylsulfathiazole and a level of x-methylfolic acid which permitted pigs to be maintained for over 100 days so that long-term observation could be made. The soy protein furnished 300 μg of folic acid per kilogram of diet. On this diet a severe anemia developed, with hematocrit values averaging 26% at 90 days. The anemia was definitely although not markedly macrocytic, the mean corpuscular volume (MCV) being 64 μ^3 compared with a normal value of 53 μ^3. No significant reticulocytosis accompanied the anemia. There was a leukopenia with the most marked reduction in polymorphic neutrophils. The marrow, aspirated from the sternum, revealed an increased proportion of nucleated red cells, which contained the macronormoblasts (megaloblastic-like cells) seen in folic acid deficiency in the pig and also a few megaloblasts similar to those found in pernicious anemia. These animals were treated at 91 days with either B_{12} or folic acid. Five animals treated with vitamin B_{12} gave reticulocyte responses averaging 13% (range 8–26%). The reticulocyte response was followed by an increase in packed cell volume (VPRC) in 4 out of 5 animals, but in no case did it reach the normal level. The sternum marrow response observed by Cartwright *et al.* (1952) consisted of an initial early increase in cellularity, at which time 90% of the cells were of the erythroid series. Two weeks later this hypercellular response had subsided and thereafter the myeloid-erythroid ratio remained constant at 0.5:1.0. The atypical megaloblasts tended to disappear although an occasional one persisted. The marrow morphology at this time was not significantly different than that previously observed in swine deficient in folic acid. In the 3 animals given vitamin B_{12} injections over 16- to 21-week periods, the packed red cell volume rose to 30%, but not to normal levels. The hematological response to B_{12} is represented in Fig. 2. Subsequent administration of folic acid caused a further increase to the normal level of 40% and production of a normal bone marrow. Animals given vitamin B_{12} gave a marked growth response compared with those not receiving vitamin B_{12}, showing that a severe vitamin B_{12} deficiency was present.

The intramuscular administration of folic acid (5 mg for 10 days then 7 mg weekly) to 5 of the doubly deficient pigs produced an average reticulocyte rise of 21%, and the packed cell volume rose to normal values within 20 to 30 days. With the disappearance of the anemia, the mean

corpuscular volume decreased to within the normal range. Leukocyte counts and especially the polymorphic neutrophils (PMN) increased transiently and then returned to the initial low values. This transient increase in PMN is similar to the large transient rise in granulocytes to higher than normal which follows the administration of folic acid to deficient rats (Daft and Sebrell, 1941). In some of the animals receiving folic acid, the packed cell volume which rose initially to 43% dropped slowly

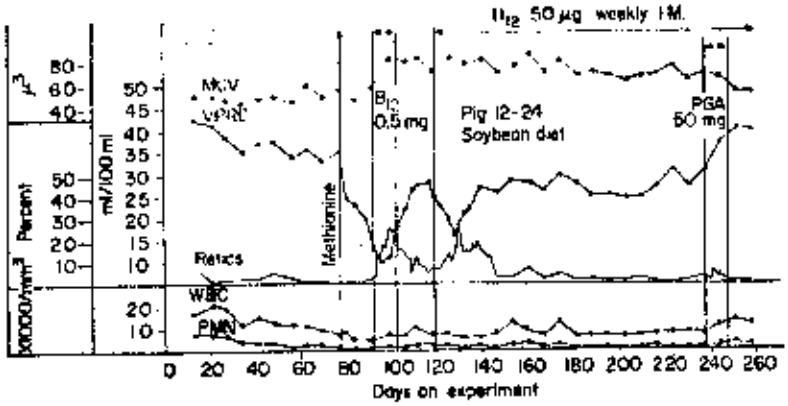

FIG. 2. Development of anemia in a pig fed a diet deficient in vitamin B_{12} and in pteroylglutamic acid (PGA) and the response to vitamin B_{12}. The animal was given an initial course of 50 μg of vitamin B_{12} intramuscularly daily for 10 days. This was followed by a reticulocytosis of 25% and a temporary increase in the VPRC from 10 to 28 ml/100 ml. After 17 days vitamin B_{12} was again given and a second response occurred. On 50 μg of vitamin B_{12} weekly, the VPRC reached a plateau at 25–30 ml/100 ml. In spite of the vitamin B_{12} therapy, the macrocytosis persisted. The administration of pteroylglutamic acid resulted in alleviation of the anemia and the macrocytosis. Methionine supplementation was begun on day 75 and continued throughout the remainder of the experiment.

MCV, mean corpuscular volume; *VPRC*, volume of packed red cells; *retics.*, reticulocytes; *WBC*, total leukocyte count; *PMN*, polymorphonuclear cells.

over a 16-week period to about 35%. At this time administration of vitamin B_{12} produced a small reticulocyte response and the hematocrit rose again to 45%. After administration of folic acid the bone marrow reverted essentially to normal. The growth response produced by folic acid alone was essentially the same as that produced by vitamin B_{12} alone. The hematological response to folic acid produced on a doubly deficient diet is shown in Fig. 3. It is interesting to note that the administration of methionine produced a moderate reticulocyte peak in view of the effect of methionine in decreasing the excretion of formiminoglutamic acid in folic acid deficiency in rats (Silverman and Pitney, 1958).

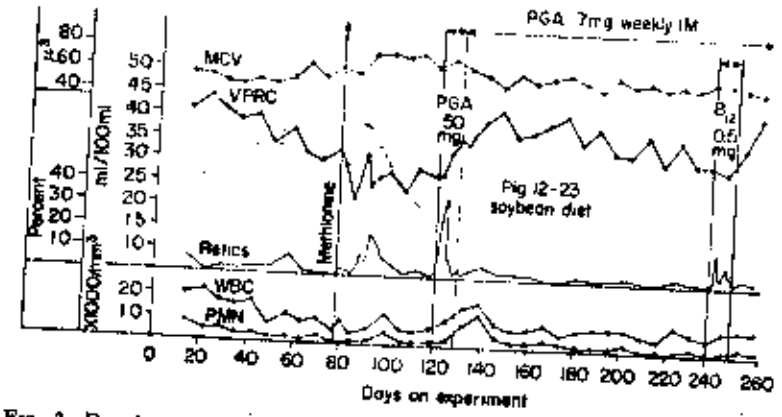

FIG. 3. Development of macrocytic anemia in a pig with the double dietary deficiency and the response to pteroylglutamic acid (PGA). After the administration of PGA the reticulocytes increased to 36%, the volume of packed red cells increased from 28 to 49 ml/100 ml and the mean corpuscular volume declined. On continued PGA therapy, anemia again developed and was then relieved by the administration of vitamin B_{12}. Methionine supplementation was begun on day 75 and continued throughout the remainder of the experiment.
For symbols see Fig. 2.

Combined treatment of these doubly deficient pigs with both folic acid and vitamin B_{12} (Cartwright *et al.*, 1952) resulted in a pronounced reticulocytosis which was greater in degree than that given by either vitamin alone. The packed cell volume returned to normal in 30 days. The mean corpuscular volume, the total leukocytes and differential counts, and the bone marrow morphology became normal. The characteristics of the

TABLE II

SUMMARY OF CHARACTERISTICS OF ANEMIAS IN SWINE ASSOCIATED WITH PTEROYLGLUTAMIC ACID AND VITAMIN B_{12} DEFICIENCIES*

Deficiency	Anemia†		Bone marrow morphology	Hemopoietic response to‡		
	Severity	Type		Folic acid	B_{12}	Folic acid + B_{12}
Folic acid	++++	Macrocytic	Macronormoblastic	++++	0	++++
B_{12}	+	Normocytic	Normoblastic	0	+	+
Folic acid + B_{12}	++++	Macrocytic	Macronormoblastic. Few megalobiasts	+++	++	++++

* Data courtesy of Cartwright *et al.* (1952).
† Key to symbols: ++++, very severe; +, mild.
‡ Key to symbols: ++++, large response; ++, moderate response; +, slight response; 0, no response.

anemias produced in deficiencies of folic acid, vitamin B_{12} and a combined deficiency of folic acid and vitamin B_{12} are summarized in Table II.

It is true that this dual deficiency in swine resembles pernicious anemia to the extent that a reticulocyte response and in some cases a rise in packed cell volume follows the administration of either folic acid or vitamin B_{12}. However, it differs in that while vitamin B_{12} will cause the packed cell volume to rise to normal in pernicious anemia, it will not do this in the doubly deficient pig.

IV. Rats

A. Folic Acid Deficiency

Rats do not develop folic acid deficiency symptoms on a folic acid-free purified diet unless an intestinal antiseptic or a folic acid antagonist is added. The feeding of purified diets containing sulfonamides (sulfaguanidine or succinylsulfathiazole) produces a severe leukopenia and granulocytopenia and in some cases a severe anemia after 3–5 weeks on the experimental diets (Spicer *et al.*, 1942; Sebrell, 1943; Kornberg *et al.*, 1943). The blood picture that has been observed in normal and folic acid-deficient rats is approximately as in the tabulation.

Rats	% Hemoglobin	Cells per mm³ Leukocytes	Granulocytes
Normal	15	10,000–15,000	2,800–5,000
Folic acid deficient	5–15	1,000–5,000	0–1,000

Examination of the bone marrow revealed varying degrees of depletion from slight to almost complete in the granulocytic series and in some cases evidence of increased erythropoiesis (Spicer *et al.*, 1942). The feeding of 10–20 μg of pure folic acid per day for 4 days increased the leukocytes and granulocytes to normal (Daft and Sebrell, 1943). In some cases there was a surge of leukocytes and granulocytes to higher than normal levels.

Rats receiving a sulfonamide have an increased susceptibility to anemia induced by frequent bleeding (Kornberg *et al.*, 1944). Although leukopenia readily develops on a sulfonamide-containing diet, anemia occurs less frequently (Spicer *et al.*, 1942; Kornberg *et al.*, 1943). The anemia induced by repeated hemorrhage is prevented by the administration of folic acid (Kornberg *et al.*, 1944). Rats fed a purified diet without sulfonamide become only moderately anemic when bled in a similar manner.

Leukopenia, granulocytopenia, and anemia have also been observed in the absence of sulfonamides on low-protein diets (Kornberg *et al.*, 1946) and on diets deficient in riboflavin and pantothenic acid (Kornberg *et al.*, 1945). On these diets folic acid gives partial hematological response or increases the response when the other missing dietary component is added. Rats raised under germ-free conditions develop folic acid deficiency in 50–70 days as evidenced by blood dyscrasia, reduced growth rate, and excretion of formiminoglutamic acid (Daft *et al.*, 1963).

The use of a folic acid antagonist produces severe deficiency symptoms rapidly. X-methylfolic acid has proved useful in producing a deficiency in the rat since it can be readily reversed by folic acid (Franklin *et al.*, 1947). By the use of 1% of x-methylfolic acid, it was found possible to produce severe granulocytopenia (200 or less per cubic millimeter) within 3 weeks in weanling rats. The effect on anemia was much less marked and animals which had severe granulocytopenia had hemoglobin levels of 10% as compared with normal values of 14–18%. Some groups in which the granulocyte level had dropped to one-third of the normal level still had normal hemoglobin values. When folic acid was added to the diet in the presence of the antagonist, a rapid increase in granulocyte count was observed which rose to higher than normal levels and then returned to normal. The effect of the antagonist and the response to added

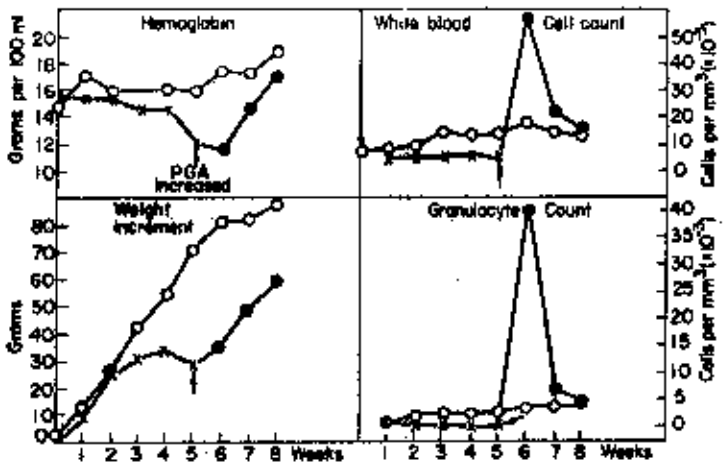

FIG. 4. Graphic representation of changes in hemoglobin, body weight, white blood cell count, and granulocyte count in 5 rats (group 12) receiving diet 1 plus 1 mg of pteroylglutamic acid per kilogram of diet (○) and in 3 rats (group 14) receiving diet 1 plus 10 gm of antagonist and 1 mg of pteroylglutamic acid per kilogram of diet (×), which was changed (arrows) to diet 1 plus 10 gm of antagonist and 100 mg of pteroylglutamic acid per kilogram of diet (●).

folic acid are shown in Fig. 4. Examination of the bone marrow showed that the proportion of nucleated erythroid cells was increased and the proportion of mature granulocytes in the myeloid was greatly decreased. The appearance of the marrow was similar to that of folic acid-deficient rats which had been fed sulfonamide-containing diets (Endicott *et al.*, 1945).

B. Vitamin B_{12} Deficiency

Vitamin B_{12} deficiency can be readily produced in rats by feeding vegetable protein diets, but no blood changes are observed unless the dams of the experimental animals are also kept on a deficient diet. Emerson (1949) reported that regular weanling rats grown on a vitamin B_{12}-deficient diet containing soybean meal and desiccated thyroid had normal red and white cell counts. The addition of vitamin B_{12} to this diet produced a 2-fold increase in growth showing that a severe vitamin B_{12} deficiency had been produced.

When females are placed on a vitamin B_{12}-deficient diet, the young which are born have hemoglobin levels and erythrocyte counts 20% and 25%, respectively, less than those from the young of mothers receiving vitamin B_{12}. There is a reduction in leukocytes, the greatest decrease occurring in the numbers of granulocytes (Newberne and O'Dell, 1959). Bone marrow from deficient animals showed active hemopoiesis and a 2- to 3-fold increase in proerythrocytic elements, most of which were pronormoblasts. It appeared that most cells were unable to complete maturation. The liver, which is part of the erythropoietic system in the infant rat, showed a reduction in hemopoietic elements. Borson *et al.* (1950) kept breeding females on a diet containing isolated soybean protein and 10% yeast as a source of water-soluble vitamins. The latter would supply 2 mg of folic acid per kilogram of diet (Toepfer *et al.*, 1951). The young which were born developed leukopenia and granulocytopenia. When the leukocyte count was below 3000 and the granulocyte count below 500 at weaning age (2–3 weeks), the survival rate was low. Red blood cell counts and hemoglobin levels were reduced, but not as severely as the granulocytes. The bone marrow showed a high degree of erythroblastic activity and myeloid activity even in animals with leukopenia and anemia (Newberne and O'Dell, 1959; Borson *et al.*, 1950). Administration of vitamin B_{12} caused rapid increase in leukocytes and granulocytes to levels higher than normal (Borson *et al.*, 1950). Red blood cell counts and hemoglobin levels rose more slowly. No reticulocyte response occurred even in extremely anemic animals. Injection of folic acid gave only a transient increase in weight and leukocyte count, despite the fact that the yeast in the basal diet furnished 2 mg of folic acid per kilo-

gram, which is well above the dietary requirement. When the litter was divided and half of the animals given a supplement of folic acid, no differences due to folic acid were observed. It is thus obvious that the blood dyscrasia cannot be prevented or cured by folic acid without vitamin B_{12}.

V. Chicks

A dietary deficiency of folic acid characterized by anemia and reduced growth rate is readily produced in chicks on a deficient diet without the use of a bacterial antiseptic or folic acid antagonist. The development of a nutritional macrocytic anemia was first reported by Hogan and Parrott (1940), and subsequently it was found that vitamin B_c (Pfiffner *et al.*, 1943) and folic acid (Jukes and Stokstad, 1947) were effective in preventing these deficiency symptoms.

Chicks placed on a purified diet deficient in folic acid develop macrocytic anemia in 3–4 weeks. There is a marked reduction in hemoglobin level, and the numbers of erythrocytes and leukocytes and of immature red cells are increased (Campbell *et al.*, 1945). The hemoglobin may be reduced from a normal level of 9% to 2–5% in severe deficiency. The injection of folic acid to deficient chicks was reported by Robertson *et al.* (1947) to increase the hemoglobin from an initial level of 2% to 8% in 10 days with a reticulocyte peak of 70% at 6 days.

Folic acid deficiency in the turkey produces a reduction in growth rate and the development of cervical paralysis, but no marked anemia has been observed (Richardson *et al.*, 1945).

Vitamin B_{12} deficiency has been produced in the chick by many investigators, but no evidence of hematological changes have been presented. Nichol *et al.* (1947, 1949) and Stern *et al.* (1952) found no reduction in the hemoglobin level of vitamin B_{12}-deficient chicks, even though the addition of vitamin B_{12} produced a 2-fold increase in growth rate. However, when chicks were injected with phenylhydrazine to induce hemolysis, the resulting anemia was greater in vitamin B_{12}-deficient chicks; and the chicks getting vitamin B_{12} regenerated hemoglobin faster after the phenylhydrazine injection was discontinued than did the deficient chicks (Stern *et al.*, 1952). In the absence of phenylhydrazine, the hemoglobin content of the chicks was the same with and without vitamin B_{12}. This indicates that vitamin B_{12} deficiency does reduce the capacity for red cell formation which becomes critical only when the stress of hemolysis is imposed.

Hemoglobin levels and red cell counts are reduced by 30% in vitamin B_{12} deficiency in chick embryos from dams on partially deficient vitamin B_{12} diets which permitted a hatchability of 45% (Hsu *et al.*, 1952). In-

jection of vitamin B_{12} into the egg increased the hemoglobin and red cell count to levels obtained by giving vitamin B_{12} in the diet of the hen.

VI. Guinea Pigs

A. Folic Acid and Vitamin B_{12} Deficiency

The guinea pig is an interesting animal for the study of the effects of folic acid and vitamin B_{12} deficiency in that it requires dietary ascorbic acid and thus is similar to monkey and man in this respect.

Folic acid deficiency can be induced in the guinea pig by simple dietary deficiency without the use of intestinal antiseptics (Woolley and Sprince, 1945; Woodruff *et al.*, 1953; Reid *et al.*, 1956).

The hematocrit is reduced from a normal level of 40% to 30–35% shortly before death, and the hemoglobin levels drop from a normal level of 14% to 10%. Corresponding decreases in number of erythrocytes occurred, and the mean corpuscular volume decreased slightly in folic acid deficiency. The total leukocytes decreased from normal values of approximately 4000 to 1700–2000 per cubic millimeter. The most marked reduction occurred in the number of granulocytes, which decreased from normal values of 1500–2000 to 600 per cubic millimeter (Reid *et al.*, 1956). Two milligrams of folic acid per kilogram of diet was found to be adequate for hemoglobin formation, but 6 mg was required (3 mg was inadequate) for white cell formation (Reid *et al.*, 1956). The requirement for maximum growth is 3–6 mg per kilogram of diet. Anemia has also been induced in guinea pigs by the administration of aminopterin (4-aminopteroylglutamic acid) (Minnich and Moore, 1948; Innes *et al.*, 1949; Girdwood, 1951).

A detailed hematological study of the peripheral blood and bone marrow in deficiencies of folic acid, vitamin B_{12}, and ascorbic acid has been made by Slungaard and Higgins (1956). They observed megaloblastosis in diets deficient in either folic acid, vitamin B_{12}, or both. Anemia developed in both deficiencies of vitamin B_{12} and of folic acid and of a combination of the two. Ascorbic acid deficiency alone produced a mild anemia in one-fourth of the animals, but no megaloblasts were observed in the bone marrow. Megaloblasts were observed in the marrow in 4 out of 8 animals deficient in folic acid, 1 out of 8 in vitamin B_{12} deficiency, and 5 out of 8 in a deficiency of both folic acid and vitamin B_{12}. When ascorbic acid deficiency was further imposed, the time required for megaloblastosis to appear was reduced.

It was also observed that diarrhea occurred in some of the deficient animals and appeared to be associated with megaloblastosis. In curative experiments 2 animals deficient in both folic acid and ascorbic acid were given separate injections of folic acid and ascorbic acid. Ascorbic acid

alone did not affect the development of severe megaloblastosis in the absence of folic acid. When folic acid was given, a reticulocyte response occurred and the megaloblastosis disappeared.

B. Effect of Folic Acid and Ascorbic Acid on Tyrosine Metabolism

A relationship has been observed between folic acid and ascorbic acid in the metabolism of tyrosine. Administration of large amounts of tyrosine to the scorbutic guinea pig produces a marked increase in the excretion of *p*-hydroxyphenylpyruvic acid, but almost none in the normal animal (Sealock and Silberstein, 1940). This increased excretion of *p*-hydroxyphenylpyruvic acid develops within 48 hours after ascorbic acid is removed from the diet and before symptoms of scurvy appear. Woodruff *et al.* (1949) found that excretion of *p*-hydroxyphenylpyruvic acid could be reduced by 25 mg of ascorbic acid per day or 5 mg of folic acid. Either supplement was effective in reducing the excretion of tyrosine metabolites, but only ascorbic acid prevented weight loss and symptoms of scurvy. Although the response of the scorbutic monkey to a load of tyrosine is similar to that of the guinea pig, folic acid is ineffective in reducing the excretion of *p*-hydroxyphenylpyruvic acid (Salmon and May, 1950, 1951).

The increase in *p*-hydroxyphenylpyruvic acid following ingestion of tyrosine in the guinea pig has been found to be the result of a rapid increase in liver tyrosine transaminase and a substrate inhibition of *p*-hydroxyphenylpyruvic acid oxidase (Knox and Goswami, 1960; Zannoni and LaDu, 1960). Ascorbic acid functions by protecting the *p*-hydroxyphenylpyruvate oxidase from substrate inhibition (Zannoni and LaDu, 1960). Folic acid functions in a similar manner by protecting the oxidase from substrate inhibition (Zannoni *et al.*, 1962). Folic acid is not active *in vitro* whereas ascorbic acid is active both *in vivo* and *in vitro*. The activity of the enzyme is not reduced on a folic acid-deficient diet, and there is no evidence that folic acid is required as a cofactor by *p*-hydroxyphenylpyruvate oxidase. It should also be noted that the amounts of folic acid required to prevent *p*-hydroxyphenylpyruvic acid excretion are large (5–20 mg/day) in comparison with the amount of folic acid needed for growth and hemoglobin formation (*ca.* 0.1 mg/day). Thus it seems unlikely that the relationship between folic acid and ascorbic acid in the metabolism of tyrosine suggests any effect of ascorbic acid on the metabolism of folic acid that may modify its hematopoietic effect.

VII. Ruminants

Folic acid deficiency has been produced in lambs fed a synthetic milk diet (Draper and Johnson, 1952). This was characterized by a marked reduction in white cells from normal levels of 6000–8000 down to 2000–

3000 per cubic millimeter, but no reduction in growth rate over an 8-week period. The hemoglobin and erythrocyte levels were unchanged, and no megaloblasts were found in the bone marrow. The addition of folic acid antagonists such as x-methylfolic acid and aminopterin accentuated the leukopenia.

Vitamin B_{12} deficiency develops when ruminants are maintained on cobalt-deficient diets. Cobalt deficiency results in loss of appetite, reduced growth rate, eventual weight loss, and severe anemia culminating in death (Underwood, 1962). Smith *et al.* (1950) reported that lambs kept for 13 weeks on low-cobalt diets (0.03 ppm of cobalt) had red cell counts and hemoglobin levels that were from one-third to one-half lower than those of controls receiving a cobalt supplement (1 mg of cobalt per day). The anemia was normocytic and normochromic. This anemia can be cured by administration of vitamin B_{12}. Administration of folic acid has no effect (Hoekstra *et al.*, 1952). The minimum effective single dose is about 100 μg of vitamin B_{12} subcutaneously (Smith *et al.*, 1951), smaller doses being ineffective. The requirement of the sheep is therefore much higher than that of the human and is more comparable on a body weight basis to that of the chick and the pig, which are roughly 1–2 μg per kilogram of body weight per day. It is also interesting that the vitamin B_{12} content of blood of deficient sheep is about 500 pg/ml and that of normal sheep 2300 pg/ml (Hoekstra *et al.*, 1952). This may be compared with the normal level of 500 pg/ml in normal humans and average levels of 40 pg/ml in pernicious anemia (Lear *et al.*, 1954).

Vitamin B_{12} deficiency has been induced in young nonruminating calves kept on synthetic milk diets containing isolated soybean protein (Draper *et al.*, 1952). Although a marked growth reduction was observed, no marked anemia occurred. The bone marrow showed a reduction in the proportion of myeloid cells and an increase in erythroid cells (Johnson, 1956). Lassiter *et al.* (1953) found a slight increase in the hemoglobin and red cell counts of vitamin B_{12}-deficient calves. This may have been due to hemoconcentration resulting from the extreme dehydration which occurred in the deficient animals.

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THE RELATION OF VITAMIN B_{12} AND FOLACIN TO THE UTILIZATION OF CHOLINE AND ITS PRECURSORS FOR LIPOTROPISM AND RENAL PROTECTION IN RATS

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THREE FIGURES

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In a previous report (Schaefer, Salmon, Strength and Copeland, '50), it was shown that the incidence and severity of renal damage in weanling rats receiving a diet low in choline and methionine were markedly reduced when the diet was supplemented with either folacin or vitamin B_{12}, and that when both were added the effect was additive. Continued studies have been made on the possible role of vitamin B_{12} and folacin in the utilization of choline or choline precursors for the maintenance of normal liver fat, prevention of renal damage, and promotion of growth. It was noted that for lipotropism both vitamin B_{12} and folacin were required, in contrast to the partial protection against renal damage provided by either vitamin B_{12} or folacin (Schaefer, Salmon and Strength, '50).

The purpose of this paper is to report the following: (1) that for lipotropism and protection against renal damage in rats, vitamin B_{12} and folacin increase the efficiency of choline, betaine, DL-methionine, dimethylaminoethanol, and methylaminoethanol; (2) that the triethylhomologue of choline appears to be an antimetabolite of choline; and (3) that vitamin B_{12} and folacin appear to be essential for the biological synthesis of choline.

EXPERIMENTAL

Weanling rats of the Alabama Experiment Station (AES) strain weighing 40 to 50 gm were placed in individual cages and uniformly grouped with respect to number, weight, sex and litter. In general, each treatment effect was determined on 4 rats per group and repeated one to 4 times. Feed and water were supplied ad libitum unless otherwise indicated. The basal diet contained extracted peanut meal 30 (50% protein) [1], sucrose 39.5, extracted casein 6 [2], salt mixture (undried) 4.4 [3], L-cystine 0.1, cod liver oil 1, and lard 19%. Vitamins were added (milligrams per kilogram of diet) as follows: thiamine HCl 2, pyridoxine HCl 2, riboflavin 4, calcium pantothenate 10, niacin 20, *i*-inositol 200, alpha-tocopherol 25, alpha-tocopherol acetate 25, 2-methyl-1,4-naphthoquinone 5. The total methionine and choline contents of the diet were 0.30% and 0.005%, respectively. In the later experiments a more purified diet was used which contained peanut meal and casein that had been further purified by 7 additional one-hour, hot 95% methanol extractions. By this procedure the choline concentration of the peanut meal was reduced from 0.17 mg per gram to 0.005 mg or less per gram.

All rats were necropsied after death or at the end of a 14-day experimental period; kidneys were examined for pathological lesions and liver fat determinations were made. Liver fat was expressed as per cent total ether-extractable material on a dry weight basis. For the determination of lipotropic activity wherever comparisons were made between treatments, it was required that maximum body weight gains be made by all groups. If not, the experiment was repeated using controlled paired feeding.

The responses to varying levels of dietary choline or choline substitutes were measured by use of three criteria; namely, body weight gain, maintenance of normal liver fat, and prevention of renal damage. In the discussion of these

[1] Engel, R. W., J. Nutrition, 1948, *36*: 739.
[2] Salmon, W. D., J. Nutrition, 1947, *33*: 155.
[3] See footnote 2.

experiments the term "choline equivalent" is used; this is based upon the hypothesis that the compounds were utilized for the biological synthesis of choline. One mole of aminoethanol, or methylaminoethanol or dimethylaminoethanol, when supplied with three, two, and one methyl groups, respectively, were considered equal to one mole of choline. For the purpose of choline synthesis, three moles of betaine HCl or DL-methionine were considered equivalent to one mole of choline. When dimethylaminoethanol was used alone, three moles were considered equivalent to two moles of choline.

RESULTS

The interrelationship of vitamin B_{12}, folacin, and choline in lipotropism

The ineffectiveness of vitamin B_{12} or folacin as lipotropic substances when fed singly to rats receiving the basal diet supplemented with 0.10% choline chloride is shown by the results in table 1. Supplementing the diet with both folacin and vitamin B_{12} reduced liver fat from an average of 43% to 18%.

Since both vitamin B_{12} and folacin were essential for the reduction of the dietary choline requirement for lipotropism, in subsequent experiments both vitamin B_{12} and folacin were either omitted or included in the diet. Supplementation of the diet with vitamin B_{12} and folacin markedly suppressed the accumulation of fat in the livers of rats fed subprotective levels of choline (table 1). In general, the liver fat of the rats fed the basal diet supplemented with 0.08% and 0.12% choline chloride and also with vitamin B_{12} concentrate and folacin was equal to that of rats receiving 0.12% and 0.16% choline chloride, respectively, without supplements of vitamin B_{12} and folacin. Since these 4 treatments supported maximum growth, protected against renal damage, and gave a marked reduction in liver fat, they were used as a basis for comparing choline to choline precursors for lipotropic activity.

Choline chloride fed at the 0.20% level was approximately as effective for the maintenance of normal liver fat as was 0.20% choline chloride plus vitamin B_{12} and folacin. The lipotropic activity of vitamin B_{12} and folacin appears to consist of a choline-sparing effect, which is manifest at subprotective levels of choline.

TABLE 1

Interrelationship of vitamin B_{12}, folacin and choline in lipotropism

DIETARY SUPPLEMENT			NO. OF RATS	AVERAGE BODY WT. GAIN	LIVER ANALYSIS		INCIDENCE OF KIDNEY DAMAGE
Choline	Vitamin B_{12}	Folacin			Fat (dry basis)	Water	
%	mg/kg	mg/kg		gm/8 wks.	%	%	%
0.04	0	0	28	19			100
0.06	0	0	16	44			69
0.08	0	0	8	49	60	54	13
0.10	0	0	8	55	43	63	0
0.12	0	0	8	56	31	62	0
0.16	0	0	4	61	17	70	0
0.20	0	0	8	63	14	70	0
0.04	2	30	12	63	56	53	0
0.06	2	30	8	55	48	57	0
0.08	2	30	8	53	28	64	0
0.10	2	30	8	56	18	64	0
0.12	2	30	8	53	17	67	0
0.16	2	30	4	62	14	66	0
0.20	2	30	8	56	12	69	0
0.10	10	0	8	59	49	57	0
0.10	0	150	8	53	39	60	0

The activity of betaine HCl and DL-*methionine in lipotropism and in protection against renal damage*

Vitamin B_{12} and folacin added to the basal diet supplemented with varying levels of betaine or DL-methionine exerted a marked sparing effect on the requirement of these compounds for the promotion of maximum growth, prevention of renal damage and lipotropism (table 2). Supplementation

of the diet with betaine HCl or DL-methionine at the 0.08% choline chloride equivalent level, without vitamin B_{12} and folacin, resulted in an incidence of renal damage of 100% (table 2) as compared to 13% for choline chloride (table 1). When the diet was supplemented with vitamin B_{12} and folacin, three moles of betaine HCl or DL-methionine were as effective as one mole of choline for protection against renal damage and lipotropism.

TABLE 2

The activity of betaine and methionine

DIETARY SUPPLEMENT			NO. OF RATS	AVERAGE BODY WT. GAIN	LIVER ANALYSIS		INCIDENCE OF KIDNEY DAMAGE
Methyl donor	Choline eq. equiv.	Folacin and vitamin B_{12}[1]			Fat (dry basis)	Water	
	%			gm/8 wks.	%	%	%
Betaine HCl	0.08	0	8	20			100
Betaine HCl	0.12	0	8	48	30	63	0
Betaine HCl	0.16	0	8	53	16	69	0
Betaine HCl	0.06	+	8	60	51	60	0
Betaine HCl	0.08	+	8	54	24	67	0
Betaine HCl	0.10	+	8	50	18	69	0
DL-Methionine	0.08	0	8	19			100
DL-Methionine	0.12	0	8	43	36	62	0
DL-Methionine	0.16	0	8	48	25	66	0
DL-Methionine	0.08	+	8	49	33	63	0
DL-Methionine	0.10	+	8	52	21	66	0

[1] Vitamin B_{12} fed at 30 μg and folacin at 2 mg per kilogram diet.

The activity of dimethylaminoethanol and methylaminoethanol in lipotropism and promotion of growth

Neither dimethylaminoethanol nor methylaminoethanol fed to rats at varying levels was equal to choline for the promotion of growth and maintenance of normal liver fat (tables 3 and 1). In the absence of vitamin B_{12} and folacin, increasing the level of methylaminoethanol or dimethylaminoethanol resulted in a depression of body weight gain. When these compounds were fed at a choline chloride equivalent level of

0.16% (without vitamin B_{12} and folacin), average liver fat values were 36 and 26%, respectively (table 3), as compared to 17% when choline was fed (table 1).

TABLE 3

The activity of dimethylaminoethanol and methylaminoethanol

DIETARY SUPPLEMENT			NO. OF RATS	AVERAGE BODY WT. GAIN	LIVER ANALYSES		INCIDENCE OF KIDNEY DAMAGE
Methyl donor	Choline eq. equiv.	Folacin and vitamin B_{12}[1]			Fat (dry basis)	Water	
	%			gm/8 wks.	%	%	%
Dimethylaminoethanol	0.12	0	6	46	28	68	0
Dimethylaminoethanol	0.16	0	6	42	26	69	0
Dimethylaminoethanol	0.40	0	8	32	24	69	0
Dimethylaminoethanol	0.08	+	4	45	46	60	0
Dimethylaminoethanol	0.10	+	8	52	32	66	0
Dimethylaminoethanol	0.20	+	6	65	18	68	0
Dimethylaminoethanol	0.40	+	4	46	17	69	0
Methylaminoethanol	0.08	0	8	31	38	67	13
Methylaminoethanol	0.12	0	8	20	36	68	13
Methylaminoethanol	0.16	0	8	10	36	70	25
Methylaminoethanol	0.20	0	8	13	29	64	25
Methylaminoethanol	0.06	+	4	51	41	64	0
Methylaminoethanol	0.09	+	4	39	38	67	0
Methylaminoethanol	0.12	+	6	48	24	67	0
Methylaminoethanol	0.20	+	8	30	20	67	0

[1] Thirty micrograms crystalline vitamin B_{12} and 2 mg folacin per kilogram of diet.

The addition of vitamin B_{12} and folacin reduced liver fat. The activity of dimethylaminoethanol was considerably greater than that of methylaminoethanol. When fed at the 0.08 or 0.10% choline chloride equivalent level, average liver fat values were 46 and 32%, respectively, for dimethylaminoethanol (table 3), as compared to 28 and 18%, respectively, for choline chloride.

TABLE 4

The toxicity of triethylcholine

DIETARY SUPPLEMENT			NO. OF RATS	AVERAGE BODY WT. GAIN	LIVER ANALYSIS		INCIDENCE OF KIDNEY DAMAGE
Triethyl choline (choline eq. equiv.)	Choline cl.	Folacin and vitamin B_{12}[1]			Fat (dry basis)	Water	
%	%			gm/2 wks.	%	%	%
0.06	0	+	4	20	42	50	50
0.08	0	+	4	32	48	58	0
0.20	0	+	4	16	33	59	25
0.20	0	0	8	2	40	58	63
0.04	0.04	+	4	60	81	52	0
0.06	0.04	+	8	54	38	61	0
0.08	0.04	+	4	44	34	63	0
0.10	0.04	+	4	38	39	59	25

[1] Thirty micrograms crystalline vitamin B_{12} and 2 mg folacin per kilogram of diet.

The toxicity of triethylcholine

The triethylhomologue of choline (triethyl-β-hydroxyethylammonium hydroxide) fed to weanling rats seemingly functioned as an antimetabolite of choline (table 4). When the basal diet was supplemented with 0.04% to 0.20% choline chloride, vitamin B_{12}, and folacin, maximum body weight gain (53 to 63 gm in two weeks) was attained (table 1); however, supplementing the diet with triethylcholine greatly arrested body weight gain and resulted in renal damage and fatty livers. The addition of choline chloride to replace part of the triethylcholine aided in promoting growth and in protection against renal damage. However, as the level of tri-

ethylcholine was increased, and the choline chloride supplement maintained at 0.04%, the body weight gain was again depressed and liver fat values remained high.

The effect of vitamin B_{12} and folacin on utilization of methylaminoethanol and methionine in lipotropism

Continued studies on the function of vitamin B_{12} and folacin in the utilization of choline precursors in lipotropism and

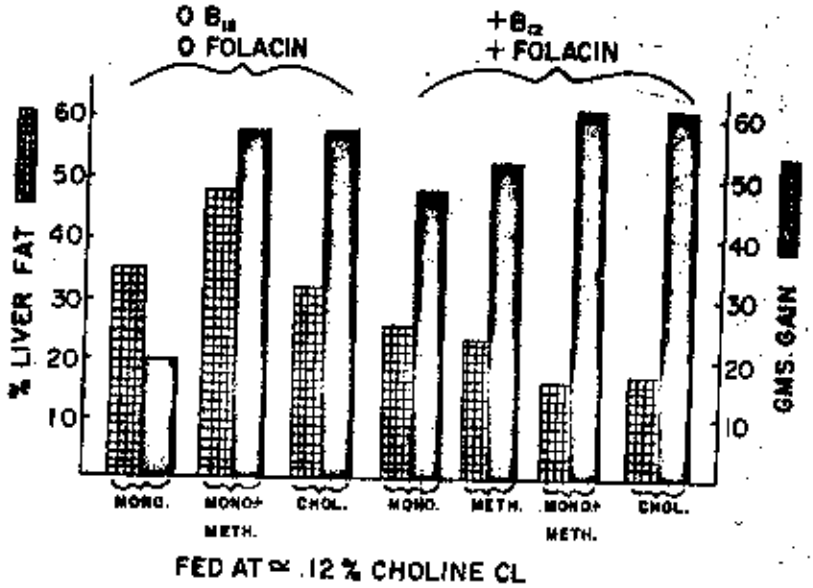

Fig. 1 The activity of methylaminoethanol and DL-methionine. Each treatment was repeated with 4 rats per group. One mole of methylaminoethanol and two moles of DL-methionine replaced one mole of choline. When methylaminoethanol was fed singly, three moles were used to replace one mole of choline. + B_{12} + folacin = 30μg of crystalline vitamin B_{12} and 2 mg folacin per kilogram of diet. Mono. = methylaminoethanol. Meth. = DL-methionine.

protection against renal damage indicated the need for a more purified diet in which the choline, choline precursors, vitamin B_{12}, and folacin content could be reduced to amounts lower than those contributed by the basal diet. In the follow-

ing experiments the more purified diet, in which the casein and peanut meal were thoroughly extracted with methanol, was used.

The original basal diet supplemented with betaine or DL-methionine at a choline equivalent level of 0.08%, plus vitamin B_{12} and folacin, completely protected against renal damage, whereas the more purified diet supplemented with the

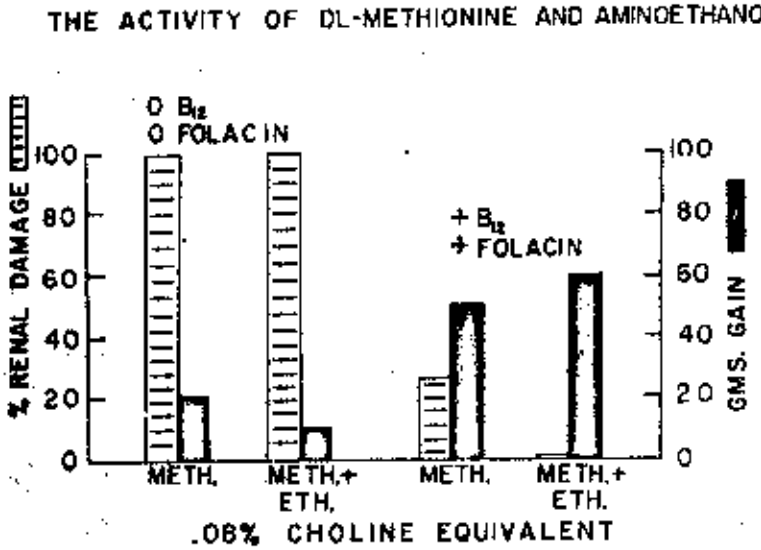

Fig. 1 Each treatment was repeated three times with 4 rats per group. Three moles DL-methionine were added in place of one mole of choline. Eth. = aminoethanol added at one mole to replace one mole of choline. Meth. = DL-methionine added at three moles to each mole of aminoethanol. + B_{12} + folacin = 30 µg crystalline B_{12} and 2 mg folacin per kilogram of diet.

same level of betaine or DL-methionine resulted in 100% and 30% renal damage, respectively.

Typical data obtained by the use of this more purified diet are illustrated in figure 1. When vitamin B_{12} and folacin were added to the diets that were supplemented with methylaminoethanol (one mole) and DL-methionine (two moles) at a choline equivalence of 0.12%, the lipotropic activity of this combination was equal to that of choline. In the absence of vitamin

B_{12} and folacin, the lipotropic activity was not equal to that of choline. These results indicate that vitamin B_{12} and folacin are involved in the biological synthesis of choline from methylaminoethanol and methionine.

The role of vitamin B_{12} and folacin in the methylation of aminoethanol by the rat

Rats fed the more purified diet supplemented with either DL-methionine, or betaine HCl, or DL-methionine (three moles)

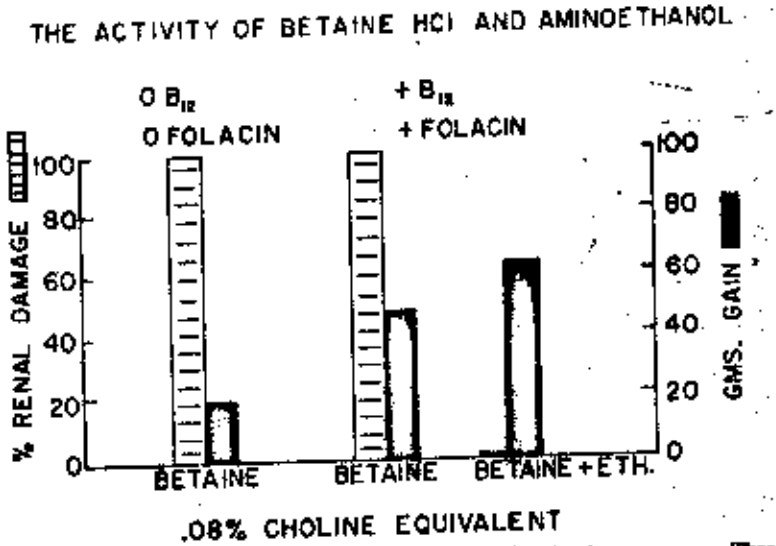

Fig. 8 Each treatment was repeated three times with 4 rats per group. Three moles of betaine HCl were added in place of one mole choline. Eth. = aminoethanol added at one mole to replace one mole choline. + B_{12} + folacin = 30 μg crystalline vitamin B_{12} and 2 mg folacin per kilogram of diet.

and aminoethanol (one mole) at a choline chloride equivalent level of 0.08%, developed 100% renal damage. Supplementation of the purified diet with vitamin B_{12} and folacin, and either DL-methionine or betaine HCl, resulted in 30 and 100% renal damage, respectively. The further addition of aminoethanol to these diets gave complete protection against renal damage and supported maximum growth. Typical data are

illustrated in figures 2 and 3. These results indicate that vitamin B_{12} or folacin, or both, are essential for the synthesis of choline from aminoethanol and methionine or betaine.

DISCUSSION

It appears that vitamin B_{12} and folacin are involved in the utilization of labile methyl, and choline and methionine precursors, for the synthesis of choline and methionine. Jukes et al. ('50) reported that vitamin B_{12} was essential for the methylation of homocystine for growth in chicks fed a methionine-deficient diet. Schaefer, Salmon and Strength ('50) reported that vitamin B_{12} was essential for the synthesis of choline from methylaminoethanol, and either methionine or betaine for prevention of perosis and production of maximum body weight gain in chicks. The present study employing rats indicates that vitamin B_{12} and folacin may function in the biological synthesis of choline from various precursors.

Since the observation of the sparing action of vitamin B_{12} on the choline requirement of rats and chicks (Schaefer et al., '49), reports by Stekol and Weiss ('50b), Welch and Sakami ('50), and du Vigneaud et al. ('50) have demonstrated the synthesis of biologically labile methyl groups *in vivo*. The data herein reported do not refute this observation; however, under the experimental conditions employed, the amount of biological synthesis of labile methyl was insufficient to meet the requirements of weanling rats for prevention of renal damage, maintenance of normal liver fat and promotion of growth.

Drill and McCormick ('49) reported that a vitamin B_{12} concentrate exerted a lipotropic effect in rats maintained on a high fat diet. In the present studies it was noted that neither vitamin B_{12} nor folacin, when added singly to the basal diet supplemented with a suboptimum amount of choline, was effective in preventing fatty livers; however, the addition of both vitamin B_{12} and folacin resulted in a marked lipotropic activity. The lack of lipotropic activity of either vitamin B_{12}

or folacin when the other is omitted from a diet deficient in choline indicates that both vitamins may have a function in the synthesis or utilization, or both, of choline. Supplementing the basal diet with vitamin B_{12}, folacin and 0.10% choline chloride maintained liver fat at a level approximately equal to that obtained when 0.16% choline chloride was fed without vitamin B_{12} and folacin. This reduction of the choline requirement by vitamin B_{12} and folacin may be due either to the biological synthesis of additional choline or to increased utilization of the choline.

Three moles of betaine HCl or DL-methionine were comparable to one mole of choline chloride for protection against renal damage and lipotropic activity when the basal diet was supplemented with vitamin B_{12} and folacin. This confirms the view of Welch ('50) that choline per se is required for these functions and that only one methyl group of betaine or the methyl of methionine may be of significance in choline synthesis.

In a comprehensive review of transmethylation reactions Jukes ('47) suggested that methylaminoethanol and dimethylaminoethanol serve as methyl acceptors and are incapable of donating methyl groups. The results of our experiments show that vitamin B_{12} and folacin greatly increased the activity of these compounds for lipotropism, prevention of renal damage and promotion of growth. Neither methylaminoethanol nor dimethylaminoethanol was equal to choline on a methyl basis; however, dimethylaminoethanol was more effective than methylaminoethanol. The increased activity of these choline precursors may be interpreted as being due to the synthesis of methyl and its utilization for choline synthesis. The results with methylaminoethanol indicate insufficient biological synthesis of methyl groups and the inability of this compound to donate methyl efficiently for the complete methylation of the aminoethanol moiety to choline.

Triethylcholine was reported to be a lipotropic agent by Channon and Smith ('36). Keston and Wortis ('46) reported

that triethylcholine is toxic to mice and that the toxicity can be alleviated by choline. Under the conditions of our experiments, triethylcholine cannot be classified as a lipotropic agent capable of replacing choline.

Stekol and Weiss ('50a) reported that triethylcholine inhibited the growth of rats and that this inhibition was alleviated by choline. Since McArthur, Lucas and Best ('47) reported evidence of the replacement of choline by triethylcholine as a component of the liver lecithins, it appears that mere incorporation of choline-like compounds into the liver lecithins does not assure lipotropic activity.

The use of a more purified diet low in choline and possible choline precursors, vitamin B_{12} and folacin, more clearly illustrated the role of vitamin B_{12} and folacin in the utilization of choline precursors. The experiments herein reported indicate that vitamin B_{12} or folacin, or both, may function in the utilization of methionine or betaine for the synthesis of choline from aminoethanol or methylaminoethanol.

In vitro studies now in progress with surviving liver slices indicate that vitamin B_{12} is involved in the synthesis of choline from methylaminoethanol and methionine.

SUMMARY

1. The choline requirement of rats for the maintenance of normal liver fat is greatly reduced by supplementing the diet with vitamin B_{12} and folacin.

2. For lipotropism and protection against renal damage in rats, vitamin B_{12} and folacin increased the efficiency of betaine, DL-methionine, dimethylaminoethanol, and methylaminoethanol as replacements for dietary choline.

3. The triethylhomologue of choline fed to weanling rats is not capable of replacing choline as a lipotropic agent.

4. Vitamin B_{12} and folacin appear to be essential for the maximum utilization of methionine or betaine for the biological synthesis of choline from aminoethanol or methylaminoethanol.

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VITAMIN B_{12} AND THE ACTIVATION OF AMINO ACIDS*

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Wagle, Mehta and Johnson [7, 8] have suggested some role of vitamin B_{12} in the protein biosynthesis. They have shown that the incorporation of labelled amino acids into protein *in vitro* has been lower in microsomes deriving from livers and spleens of vitamin B_{12} deficient rats than in those deriving from normal animals. The addition of vitamin B_{12} has risen the rate of amino acids incorporation in preparation deriving from the vitamin deficient rats. It has been stated later that the rate of amino acids activation has been lowered by as much as 40% when an antivitamin, namely the vitamin B_{12} anilide, was added. The experiments performed by these authors to study the localization of vitamin B_{12} in the cells have revealed that it occurs mainly in microsomes and in the supernatant collected during centrifugation of the microsomes. It has been stated on fractionation of the supernatant that 70% of the vitamin present in it has been precipitated at pH 5 with the amino acid activating enzymes. The preliminary fractionation of "pH 5 enzymes" has proved, that nearly whole vitamin B_{12} has been precipitated with the protein at the ammonium sulphate saturation 0.4 to 0.6. This fraction represents a mixture of the crude amino acid activating enzymes. Basing upon these results Wagle, Mehta and Johnson [8] have suggested that vitamin B_{12} plays some role in the amino acids activation. These reports have been criticized recently [1, 4], but all the experiments, both those supporting the hypothesis and those contradicting it, have carried out on the only slightly purified enzymatic preparations. Therefore, we have decided

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to isolate the tryptophan activating enzyme up to the stage of its high purity and to follow the vitamin B_{12} contents in various fractions obtained during the process of the purification.

EXPERIMENTAL

The tryptophan activating enzyme was isolated from the ox pancreas acetone powder. The method described in the following appears to be more convenient than that referred to by Davie, Koningsberger and Lipmann [2], since it allows to obtain a powder retaining its activity on storage and gives a high yield of active enzyme.

Methods. The activity of the tryptophan activating enzyme was estimated by hydroxamic method [2]. Glycine hydroxamate prepared according to Safir and Williams [6] was employed as the standard solution. The accuracy of the method in our experiments remained within the limits of $\pm$ 5%. The vitamin B_{12} content was estimated by means of the Ford's microbiological method using *Ochromonas malhamensis* [3]. This method has been chosen for its high sensibility and almost absolute specificity towards cyanocobalamine.

The protein content was estimated by the biuret method [5].

Acetone powder. 500 g of fresh pancreas, free from fat and conjunctive tissue was minced in a mincer with holes of about 1.5 mm in diameter. The tissue minced in this manner could be subsequently well dehydrated and yielded a powder of long stability. The minced pancreas was treated with 3 l of acetone cooled to $-15°$ and the extraction was carried out for 15 min at about 0°. Then the suspension was quickly filtered under *vacuo* and the extraction twice repeated with 2 l of cold acetone each time. The powder was washed with 500 ml of cold acetone on the Büchner funnel and dried quickly at room temperature with a stream of cold air. The powder stored in vacuum dessicator over $CaCl_2$ at 4° retained its activity for several months.

Fractionation. All the manipulations were performed at 0°. 50 g of the acetone powder were extracted with 700 ml of 0.05M-KCl for 20 min and stirred occasionally during this extraction. The suspension was centrifuged for 15 min at 4000 *g*, the sediment discarded while the extract was collected and the activity of the tryptophan activating enzyme was determined in it, as well as the vitamin B_{12} content (Fraction I). The results obtained in this fraction from different acetone powders varied from 0.4 to 1.5 μmoles of the hydroxamate formed per 1 mg of protein per hour. The vitamin B_{12} content in the same fraction ranged from 0.6 to 2.1 mμg per 1 mg of protein.

To further purification the extract was acidified with 2 N-acetic acid to pH 4.55 and centrifuged at 4000 *g*. Vitamin B_{12} was estimated in the supernatant. In amounted for about 20% of the quantity found in the extract from acetone powder. The remaining 80% was precipitated with the amino acid activating enzymes at pH 4.55. This precipitate was then dissolved in 200 ml of 0.2 M-Tris buffer, pH 7.6, and the insoluble residue discarded after centrifugation. The supernatant, called Fraction II, contained only 30% to 60% of the vitamin B_{12} that had been extracted from acetone powder, since some part of the vitamin was discarded with insoluble proteins. The total enzymatic activity of this fraction was lower than that of Fraction I, the difference being 30% to 45% while the specific activity was in certain cases more than twice that found in Fraction I.

Solid ammonium sulphate was added to the Fraction II to give 0.45 saturation, the resulting precipitate was removed by centrifugation at 30 000 *g* for 10 min and discarded. The supernatant was acidified with 1 N-acetic acid to pH 4.55, centrifuged at 30 000 *g*, the precipitate dissolved in 20 ml of Tris buffer, pH 7.6, and the insoluble residue discarded (Fraction III). The loss of the total activity during the preparation of the Fraction III was 20% to 50%, as compared with Fraction II, but the specific activity was 8 times higher. It was rather a considerable increase when compared to that observed during the passage from the first fraction to the second.

Saturated ammonium sulphate solution was then added to the Fraction III to reach up 0.45 saturation. The resulting precipitate was discarded after centrifugation at 30 000 *g* for 10 min and solid ammonium sulphate was added to the supernatant up to 0.6 saturation. The obtained precipitate was centrifuged in the similar manner and dissolved in 10 ml of water (Fraction IV). The loss of total activity at this step was 45% whereas the specific activity increased about 3 times and the vitamin B_{12} content 2 times, as calculated per 1 mg of protein.

Fraction IV was submitted to dialysis for 2 hours against 4 l of distilled water stirred continuously. The precipitate being discarded, the liquid was acidified with 0.1 N-acetic acid to pH 4.8 and centrifuged at 30 000 *g* for 5 min. Thus obtained precipitate contained the very active enzyme but its specific activity was somewhat lower than that of the next fraction. The supernatant was acidified with 0.1 N-acetic acid to pH 4.55 and centrifuged at 30 000 *g* for 10 min. The resulting precipitate was suspended in 2 ml of water and some few drops of 0.1 N-NaOH were added to give pH 6.5. The insoluble residue was removed by centrifugation and discarded. The supernatant was the Fraction V. The loss of activity was sometimes considerable at this step of the procedure

and reached up even as much as 80% of the total activity found in the Fraction IV, but the specific activity increased strongly, and so did the vitamin B_{12} content as calculated per 1 mg of protein. Fraction V contained the highly purified enzyme which showed one main fraction and only some unsignificant contaminations on acetylocellulose electrophoresis. The electrophoresis was carried out in 0.05 M-veronal buffer, pH 8.5 for 3 hours, 0.6 mA being applied on 1 cm of the strip. We have succeeded to crystallize the enzyme out of Fraction V using ammonium sulphate (Fig. 1). The enzyme has been also crystallized at its isoelectric point reached up with acetic acid. It was, however, impossible to recrystallize it on account of too small amounts of the enzyme. The specific activity of the crystalline enzyme was the same as that found in Fraction V from which it had been crystallized.

Fig. 1. Crystals of tryptophan activiting enzyme

The results of two typical fractionations have been gathered in Table 1. The amount of vitamin B_{12} as calculated per 1 mg of protein increased along the purification of the enzyme. Thus it might look likely that the vitamin was bound to the tryptophan activating enzyme. When one considers, however, that the molecular weight of vitamin B_{12} is 1300, while that of the enzyme is 20 000 to 30 000, according to Lipmann *et al.* [2] one can calculate that there should be one molecule of the vitamin for every 4000 to 6000 molecules of the enzyme. If the equimolar ratio vitamin-enzyme were assumed, the molecular weight of the enzyme would have to be about 130 000 000. There is no doubt, however, that some considerable concentration of the vitamin is obtained during the purification of the enzyme.

Another suggestion arises that the enzyme has some specific ability to adsorb the vitamin selectively. To study this hypothesis the extract

Table. 1

Vitamin B_{12} content in diffetent fractions during purification of the tryptophan activating enzyme isolated from ox pancreas

The figures are the results of two experiments *a* and *b*. The fractionation was carried out on 50 g of acetone powder.

Fraction	Experiment	Total protein (mg)	Total activity (μmole of hydroxamate/hr)	Total vitamin B_{12} (mμg)	Specific activity (μmole of hydroxamate/mg of protein/hr)	Vitamin B_{12} (mμg/mg of protein)
I	*a*	19 380	14 922	17 823	0.77	0.93
	b	11 480	17 000	24 820	1.48	2.14
II	*a*	8 875	8 342	10 910	0.94	1.26
	b	3 740	12 144	8 380	3.25	2.24
III	*a*	508	4 014	1 764	7.90	3.47
	b	676	9 193	2 410	13.60	3.57
IV	*a*	227	2 275	1 180	20.00	5.30
	b	300	8 928	2 285	31.00	7.65
V	*a*	29.0	1 363	180	47.00	6.30
	b	43.0	1 870	379	43.50	9.05

of acetone powder containing itself about 20 μg of vitamin B_{12} was treated with the equal amount of the vitamin, and the whole fractionation procedure was performed on the so enriched extract. The contents of vitamin B_{12} in individual fractions varied with the same range of values as they did in the corresponding fractions of the extract without the vitamin added. Thus the last experiment did not provide any evidence for the selective adsorption of vitamin B_{12} on the tryptophan activating enzyme. Yet it did not exclude this possibility, since the enzyme might be completely saturated with the vitamin present in preparation originally. It was possible, too, that vitamin B_{12} was bound to other proteins accompanying the enzyme, since electrophoresis showed that even the preparations of the highest purity still contained some contamination of heterogeneous proteins. Our results, when calculated as the total vitamin B_{12} content in the purified enzyme, showed that the vitamin occurred in amounts which could be considered as contamination only.

These results are not the only ones contradicting those of Johnson et al. [7, 8], since Arnstein and Simkin [1] when working like Johnson on rats have not found any difference between the rate of the protein synthesis in the vitamin B_{12}-deficient rats and the normal ones. Microsomes isolated from the livers of the vitamin B_{12}-deficient rats and from those deriving from the normal ones have shown the same rate of incorporation of ^{14}C-labeled amino acids. The addition of vitamin B_{12} to the former preparations did not influence the rate of amino acids activation nor that of their incorporation into microsomes.

It results apparently, from our experiments, that vitamin B_{12} is not any component of the tryptophan activating enzyme. Hence Johnson's suggestion on the direct participation of the vitamin in the amino acids activation does not appear to be justified.

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SUMMARY

The tryptophan activating enzyme was isolated from the ox pancreas acetone powder. The amount of vitamin B_{12} as calculated per 1 mg. of protein increased on purification and reached its highest value in crystalline enzyme. However, when molecular weight of vitamin B_{12} was considered, as well as that of the enzyme, it resulted from the calculations that one molecule of the vitamin corresponded to 4000–6000 molecules of the enzyme. Thus, it became apparent that vitamin B_{12} did not take any direct part in the tryptophan activation.

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SERUM VITAMIN B12 LEVELS IN PREGNANT WOMEN

BY

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SERUM vitamin B12 levels in pregnant women at term have been shown to be lower than non-pregnant women in the child bearing age (Heinrich, 1954; Boger *et al.*, 1956; and Lawrence and Klipstein, 1967). Furthermore, serum vitamin B12 levels fall progressively throughout pregnancy (Okuda *et al.*, 1956; Luhby *et al.*, 1961). It has been noted also that the serum vitamin B12 levels in cord blood are considerably higher than those of the maternal blood at term (Killander and Vahlquist, 1954; Baker *et al.*, 1958, and Zachau-Christiansen *et al.*, 1962).

Experiments with rats have shown that severe vitamin B12 depletion in females is associated with reproductive abnormality and debilitated offspring. It has also been demonstrated that vitamin B12 significantly reduces the number of stillbirths in experimental animals. This aspect has been reviewed by Nesbitt and Chow (1958). The present study examines serum vitamin B12 levels throughout pregnancy and relates the initial visit level to the outcome of pregnancy.

PATIENTS

The serum vitamin B12 level was estimated in 155 women at their initial visit, at 32 and 36 weeks and at term. The time of the initial visit varied from 10 to 24 weeks with a mean of 17 weeks. The blood sample at term was usually taken two or three days after delivery. Approximately 20 per cent of patients attending the antenatal clinic in the Rotunda Hospital are primigravidae and the only concession to selection was to ensure that a similar proportion was included in the survey. By full term the number of estimations representing the original 155 patients had diminished to 133 because of miscarriages, premature deliveries and home confinements. The ages of the patients ranged from 18 to 43 years, with a mean of 28 years. The mean parity of 114 multiparous patients included in the survey was 3·1. In 53 cases the serum vitamin B12 level was also estimated three months after delivery. Blood samples were taken from a further 117 patients at random prior to the 20th week.

The survey was undertaken in 1964–65. The employment of the husbands of the women questioned was as follows: tradesmen or similar occupation 27 per cent; semi-skilled workers 15 per cent; unskilled labourers 43 per cent and unemployed or casual workers 15 per cent. The average weekly income of the family was approximately £10 15s. 0d. and the average income per head £2 10s. 0d. The diet of patients visiting the Rotunda Hospital out-patients department has been reviewed by Browne and Callaghan (1964) who concluded that the average diet of pregnant women attending the out-patient department contained too much carbohydrate and insufficient protein, calcium, iron, thamine and riboflavin.

METHODS

Serum vitamin B12 levels were estimated using *Lactobacillus leichmannii* as the test organism (Temperley and Collery, 1965). The method was based on that of Spray (1955). The range and mean of 118 normal subjects was 125 to 1,050 pg./ml. and 472 pg./ml. respectively.

TABLE I

Serum Vitamin B12 Levels at Various Stages of Pregnancy

Stage of Pregnancy (weeks)	No. of Cases	Serum Vitamin B12 (pg./ml.)		
		Range	Mean	S.E. of Mean
First visit	154	50–950	239	12·0
32	152	40–480	164	6·1
36	134	50–475	162	7·0
40	133	20–510	165	7·3
Non-pregnant	36	205–1,025	454	31·3

RESULTS

Fall of Serum Vitamin B12 Levels Throughout Pregnancy

The range, mean and standard error of the mean serum vitamin B12 levels at the initial visit, at 32 and 36 weeks and at full term are shown in Table I. The mean serum level fell from 239 pg./ml. at the initial visit to 164 pg./ml. at 32 weeks. From this time onwards little change occurred in the mean figures. The results compare with a range and mean in 36 non-pregnant women in the child bearing age of 205 to 1,025 pg./ml. and 454 pg./ml. respectively.

Of the original 155 patients the serum vitamin B12 level was estimated three months after delivery in 53 instances. These results were compared with the levels of the same 53 patients at their initial visit to the antenatal clinic. The mean serum vitamin B12 level three months after delivery was 338 pg./ml. (S.E. $\pm$ 20·5) compared with a mean level of 224 pg./ml. (S.E. $\pm$ 17·6) at the initial visit. The difference between these results was statistically significant ($p<0·01$).

Because of the suspected sharp fall in the serum vitamin B12 levels relatively early in pregnancy, the results at the initial visit were grouped into 12 weeks or less, 13 to 16, 17 to 20, and 21 to 24 weeks. The numbers of patients included in these groups were 17, 59, 56 and 22 respectively and the mean serum vitamin B12 levels were 316, 240, 223 and 211 pg./ml. respectively.

The range of serum vitamin B12 levels in 33 patients with untreated pernicious anaemia was 0 to 110 pg./ml. (Temperley and Collery, 1965). Serum vitamin B12 levels were found to be in this range in 11 patients at their first visit (7·3 per cent), in 37 patients at 32 weeks (24·3 per cent), in 34 patients at 36 weeks (25·4 per cent), and in 41 patients at full term (30·9 per cent). Of the 52 patients investigated three months after delivery, two patients had a level of less than 110 pg./ml. Both patients had a low level at the first visit (80 and 62 pg./ml.). The birthweight of each infant was six pounds (2·72 kg.). No relationship was noted between the age or parity of the mother and serum vitamin B12 levels.

Fetal Serum Vitamin B12 Levels

Cord blood serum vitamin B12 levels were compared with maternal levels at various stages of pregnancy in 53 instances (Table II). In general mean cord levels were higher than maternal levels at all stages of pregnancy. However, in the case of cord levels grouped under less than 200 pg./ml. the mean cord result was less than the maternal level at the initial visit. There was a relationship between cord and maternal serum vitamin B12 levels throughout pregnancy; low mean cord levels were associated with low maternal levels and high cord levels with high maternal levels.

TABLE II

Comparison of Cord and Maternal Serum Vitamin B12 Levels in 53 Instances

Cord Serum Vitamin B12 (pg./ml.)	Mean Maternal Levels (pg./ml.)				Mean Cord Level (pg./ml.)
	First Visit	32 Weeks	36 Weeks	Full Term	
<200	181	110	126	123	148
200–299	207	164	193	176	252
300–399	215	179	174	210	341
400 and >400	279	214	209	213	521
All results	222	166	176	176	313

TABLE III

Relationship of Initial Visit Maternal Serum Vitamin B12 Level With Outcome of Pregnancy

Clinical Events	Serum Vitamin B12 Level (pg./ml.)				Mean Serum Vitamin B12 Level (pg./ml.)
	<150	150–199	200 and > 200	Total	
Abortion	1	0	3	4	350
Deadborn, macerated	2	2	2	6	200
Neonatal death	3	2	2	7	167
Prematurity	12	3	4	19	167
Congenital abnormality	2	0	1	3	162
Toxaemia	4	0	9	13	293
Accidental haemorrhage (toxaemic)	0	0	3	3	311
Unclassified accidental haemorrhage	4	2	2	8	164
Twins	4	0	2	6	121
Total number of patients in each vitamin B12 group	51	40	157	248	262

Serum Vitamin B12 Levels at the Initial Visit

The relationship of the serum vitamin B12 level at the initial visit with obstetrical complications and fetal loss is listed in Table III. Results were grouped into three; levels less than 150 pg./ml. an intermediate group between 150 and 199 pg./ml. and those greater than 200 pg./ml. A total of 248 patients who attended before the 20th week was assessed. These included 131 of the original group of 155 mothers and a further 117 who had only a blood sample collected at the initial visit.

Prematurity

There was a disproportionally large number of premature infants (birthweight 5 pounds 8 ounces (2·5 kg.) or less) born to mothers whose serum vitamin B12 level was less than 150 pg./ml. (Table IV). It was unlikely that this distribution occurred by chance ($\chi^2 = 24{\cdot}1$, $p<0{\cdot}01$). Some details of the premature infants are shown in Table IV. Macerated fetuses and induced deliveries were not included. The mean gestation period of the 19 premature infants as judged by the first day of the last menstrual period was 36 weeks.

Neonatal Death

Of the seven neonatal deaths six were associated with prematurity and one with congenital abnormality. The distribution of maternal serum

TABLE IV

Serum Vitamin B12 Levels at the Initial Visit of Patients Delivered of Premature Infants

Case No.	Serum Vitamin B12 (pg./ml.)	Factors Relating to Prematurity	Duration of Pregnancy (weeks)
1	122	Accidental haemorrhage (unclassified)	37
2	118	Accidental haemorrhage (unclassified)	32
3	430	Rhesus negative haemolytic disease	36
4	135	Anencephalus	33
5	123	None	33
6	200	None	34
7	67	None	36
8	140	Mild toxaemia	37
9	117	None	37
10	195	None	35
11	115	Rhesus negative with antibodies, deadborn	32
12	187	None	32
13	225	Intestinal malrotation	37
14	300	None	37
15	137	None	40
16	95	Twins, toxaemia	35
17	195	Twins	30
18	141	Twins	38
19	130	Twins	35
Mean	167		

vitamin B12 levels and the mean serum vitamin B12 level of 167 pg./ml. was therefore similar to that of the premature group (Table III).

Deadborn

This group included three fresh and three macerated stillbirths; two were associated with rhesus incompatibility, one with prolapsed cord, one with accidental haemorrhage. The mean serum vitamin B12 level of this group was 200 pg./ml.

Abortion

Four initial estimations were available from patients who had abortions. The results individually were 425, 443, 431 and 100 pg./ml. This group is not representative as the mean time of the initial visit was 17 weeks; many abortions would have occurred before this stage of pregnancy.

Congenital Abnormality

There was one case of idiopathic hydrops fetalis (rhesus negative, no antibodies), one of malrotation of the intestines and one of anencephalus included in this group. The initial visit serum vitamin B12 level of the mother who was delivered of the anencephalic infant was 135 pg./ml. The possible importance of this led to the estimation of the levels of further samples of serum from 11 mothers at or before the 16th week who were delivered of anencephalic infants. The range and mean of the 12 results were 135 to 457 pg./ml. and 283 pg./ml. respectively.

Toxaemia and Antepartum Haemorrhage

The mean initial serum vitamin B12 of toxaemic mothers was 293 pg./ml. which compares with the mean of 267 pg./ml. for the total 268 initial visits. Accidental haemorrhage with toxaemia was also associated with levels at or above the total mean. However, the mean level for unclassified accidental haemorrhage was 164 pg./ml. There was a disproportionate number of unclassified accidental haemorrhages in mothers with an initial level of less than 150 pg./ml. (Table III). However, the possibility that the distribution occurred by chance cannot be outruled ($\chi^2 = 5{\cdot}97$, $p = 0{\cdot}05$). Two patients with placenta praevia had levels of 218 and 215 pg./ml.

Twins

The mean serum vitamin B12 level of six mothers who were delivered of twins was 121 pg./ml. Of the twelve infants, seven were premature by weight and two of the premature infants were stillbirths. There was one case of accidental haemorrhage and one mother had toxaemia. There was a disproportionate number of results less than 150 pg./ml. (Table IV). It was unlikely that this distribution occurred by chance ($\chi^2 = 8{\cdot}58$, $p<0{\cdot}02>0{\cdot}01$).

Discussion

The present study of 155 women confirms the fall of serum vitamin B12 levels with progression of pregnancy. If the mean serum vitamin B12 level prior to conception was similar to that of a group of non-pregnant women in the child-bearing age then the major part of the fall has occurred by approximately 17 weeks (Table I). This is substantiated by the observation that the mean level three months after delivery was 338 pg./ml. in 53 women compared with 224 pg./ml. at the first visit. There is a less rapid fall from the 17th week to the 32nd week when the mean level remains constant until term.

Reference has been made to higher vitamin B12 levels in cord compared with maternal blood at term. Chow, Barrow and Ling (1951) have shown that when radioactive vitamin B12 was administered orally to pregnant rats 60 per cent of absorbed vitamin B12 was concentrated in the fetus. It has also been demonstrated that when crystalline vitamin B12 is given to human mothers six hours before parturition fetal serum vitamin B12 levels are higher than maternal levels at delivery (Nesbitt and Chow, 1958). These observations have led to the assumption that the fall in maternal serum vitamin B12 levels is due to fetal utilization. The findings of the present communication suggest that there is greater utilization of vitamin B12 by the fetus or the placenta in the early months of pregnancy, and that by 32 weeks demands by the fetus had lessened to the extent that while the average mother was incapable of replenishing her stores a balance between intake and utilization had been reached.

Utilization by the fetus as a cause of low maternal serum vitamin B12 is further substanti-

ated by the present observation of particularly low levels in mothers who were ultimately delivered of twins. While the weight of evidence points to the above conclusion, Lawrence and Klipstein (1967) have recently suggested that the serum of pregnant women may contain an inhibitor to the growth of test organisms used in the assay of vitamin B12.

The mean maternal serum vitamin B12 levels at or near delivery was 56 per cent of the mean cord level in 53 paired results. This relationship is similar to many reported figures listed by Zachau-Christiansen and his co-workers (1962). It was noted that the nursing staff tended to ignore collection of cord blood following an obstetrical emergency or premature delivery. These omissions brought an element of selectivity into this aspect of the study. It was, therefore, not possible to compare cord levels with abnormalities associated with pregnancy. Low cord levels were associated with low cord levels at all stages of pregnancy (Table II). It seems likely, therefore, that avitaminosis B12 may occur in the fetus of a mother depleted of vitamin B12.

There is little reported evidence that low maternal serum vitamin B12 levels associated with pregnancy itself have an effect on the human fetus. Megaloblastic anaemia of infancy secondary to maternal pernicious anaemia has been observed (Lampkin *et al.*, 1966) and Baker and his co-workers (1962) have reported intermediate megaloblastic marrow change responsive to vitamin B12 in an infant of a mother with avitaminosis B12. There can be no doubt from reported evidence that in animals all degrees of reproductive failure can be observed from dietary restrictions of vitamin B12.

However, the nutritional stress to which these animals are subjected must seldom be obtained in women. Low birthweight (Jones *et al.*, 1955) and an increased incidence in the number of stillbirths and neonatal deaths (Sure, 1951; Lepkovsky *et al.*, 1951) have been demonstrated in the young of rats fed a vitamin B12 deficient diet.

As developmental abnormalities are likely to originate in the first months of pregnancy, and because of the present observation that the fall of maternal serum vitamin B12 levels seemed particularly rapid in this period, levels prior to the 20th week were related to the outcome of pregnancy. In a randomly selected group of 248 pregnant women 19 were delivered of premature infants as judged by weight. Of these 12 had serum levels of less than 150 pg./ml. at their initial visit. The duration of pregnancy was, in all cases but one, less than term. (Table IV) Low levels were, therefore, primarily associated with prematurity of delivery rather than retardation of growth. It is of interest that the birthweight of the infant born to a mother with pernicious anaemia reported by Lampkin and his co-authors (1966) was 5 pounds 8 ounces (2·5 kg.). Zuelzer and Rutzky (1953) have reported megaloblastic anaemia responsive to vitamin B12 in a premature infant.

Paucity of numbers prevented an assessment of the relationship of serum vitamin B12 levels with many abnormalities of childbirth. No comment is therefore made on abortions and stillbirths. It was possible to estimate serum vitamin B12 levels at or before the 16th week of mothers who were delivered of anencephalic infants; eleven sera were obtained from a source outside the present survey. The mean result was 283 pg./ml. which is slightly higher than the overall mean of the randomly selected group. However, it might be expected that levels at or before 16 weeks would be somewhat higher than those prior to 20 weeks.

Results from toxaemic mothers were distributed in approximately equal proportions between the relatively high and low serum vitamin B12 levels. There was a difference in distribution between the results in patients who had an unclassified accidental haemorrhage compared with those who bled in association with toxaemia; the former had predominantly low serum vitamin B12 levels whereas the latter had higher levels.

The pattern of fall of maternal serum levels suggests that the fetal requirement for vitamin B12 is less in the viable fetus than at earlier stages of fetal life. The negative maternal balance, as judged by serum levels, coincides with the most rapid phase of organ development. The association of low maternal vitamin B12 levels at the initial visit with premature delivery suggests that vitamin B12 may be an essential requirement for the proper development of the placenta. It is clear, however, that only tentative speculations can be made from the present evidence.

Summary

Mean serum vitamin B12 levels in 155 pregnant women fell from the initial visit to approximately the 32nd week. From this time onward to term no further fall in the mean level was observed. In 53 instances the mean serum vitamin B12 level three months after delivery was 338 pg./ml. compared with a level at the initial visit of 224 pg./ml. The mean level was 454 pg./ml. in 36 non-pregnant women in the child-bearing age. By inference these results suggest that the major fall in maternal levels occurs in the first four or five months of pregnancy.

Cord serum vitamin B12 levels were in general higher than the maternal levels throughout pregnancy. Low cord levels were associated with low maternal levels.

Maternal serum vitamin B12 levels obtained prior to the 20th week were compared in 248 cases with the outcome of pregnancy. There was an association between low levels and premature delivery. The mean serum vitamin B12 level was particularly low in twin deliveries.

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Degeneration of the Peripheral and Central Nervous System in Vitamin-B_{12}-Deficient Monkeys

Following on the report of OXNARD and SMITH[1] on reduced serum-vitamin-B_{12} levels and neurological degeneration in captive monkeys, further qualitative and quantitative investigations have been carried out, with particular reference to the peripheral nerve lesions and to the effect of treatment.

Materials and methods. 43 monkeys in the colony of the Anatomy Department, University of Birmingham, were studied. There were 40 rhesus monkeys (*Macaca mulatta*), 2 patas monkeys (*Erythrocebus patas*), and 1 baboon (*Papio anubis*); 2 were males and 41 were females of which 10 were pregnant. The animals were grouped according to duration of captivity and diet: group I comprised 12 monkeys kept in captivity from 11 months to 10 years on a standard vegetarian diet; group II comprised 14 monkeys captive from 11 months to almost 19 years, originally fed vegetarian diets but subsequently given a series of injections of vitamin B_{12} followed by a normal diet for periods that varied from 6 months to 4 years; and control group III comprised 17 recently captive monkeys given vitamin B_{12} since arrival in the colony, 14 animals having been in captivity for less than 1 month, and the other 3 animals for 6, 7 and 15 months respectively. The amounts of vitamin B_{12} in the serum were estimated in 38 cases by the bioassay technique with *Euglena gracilis*; OXNARD[2] found the mean total level to be 271 µg/ml in recently captive monkeys. In group I 10 animals had deficient levels of the vitamin in the serum (mean total level 79 µg/ml); in group II 12 animals had high serum levels (mean total > 900 µg/ml); and in group III the serum levels were normal, except for 2 pregnant monkeys that had low readings.

Results. The results are given in the Table.

A high incidence of histological lesions was found in animals of groups I and II and 5 animals showed overt paralysis (see Table). It should be noted that this does not represent the true incidence of paralysis in deficient animals, which is much lower; paralysed animals were selected because of the paralysis and in order to try and assess the effect of treatment. In the spinal cord the changes resembled those of human subacute combined degeneration, and were more severe in the paralysed monkeys; cerebral lesions were found in 9 animals. Frozen sections of the sciatic and popliteal nerves showed sudanophilic degeneration in 21 animals (the median and ulnar nerves were normal in all 43 cases). Segmental demyelination, usually with remyelination, was seen in teased peripheral nerve fibres in every animal that showed sudanophilic degeneration (see Figure); in a few animals there was also axonal (wallerian) degeneration.

In group III minimal lesions were found in 4 animals. Two of these were pregnant and had low serum-vitamin-B_{12} levels (pregnancy depresses the serum vitamin B_{12} in monkeys; OXNARD[3]) and a third animal had a low-normal level; though the previous dietary history of these 3 animals was unknown, their nervous systems could have been affected before they arrived in captivity. The fourth animal had been captive for 15 months (more than twice as long as any other animal in group III) and

[1] C. E. OXNARD and W. T. SMITH, Nature *210*, 507 (1966).
[2] C. E. OXNARD, Nature *207*, 1888 (1964).

Incidence of neurological lesions in groups I–III

Group (total No. of animals)	Functional status		Histological findings						
	Normal	Paralysed	No. of animals with lesions	Distribution of lesions					
				Cerebrum	Cerebellum	Brain stem	Spinal cord	Posterior nerve roots and/or posterior root ganglia	Peripheral nerves (lower limbs)
I (12)	11	1	12	3	1	3	12	3	10
II (14)	10	4	13	2	1	1	11	6	11
III (17)	17	0	4 (slight)	0	0	0	2	1	3

The nerve roots and the posterior root ganglia were not examined in 2 cases in group I, neither was the brain-stem in one of these cases.

(a) A dissected nerve fibre showing an incompletely remyelinated short segment (arrow). Osmium stained. × 100. (b) A dissected nerve fibre showing a widened node of Ranvier and part of a demyelinated internode in which the Schmidt-Lantermann incisures are clearly seen. Osmium stained. × 720. (c) Two dissected nerve fibres. One of these shows part of a normal internode and 2 thin remyelinated segments of reduced internodal length. Phagocytic cells adhere to the surface of the fibres. Osmium stained. × 250.

by an oversight its serum level was not examined; previous studies[1] showed that reduced levels occur rapidly and neural lesions could result in this period if, as can happen, the animal had not been receiving its proper diet.

Nerve fibre counts. Group I (vitamin B_{12}-deficient animals), group II (long stay non-paralysed treated animals), and group III (recently captive animals), showed similar average mean fibre density counts/mm² of intraperineurial area in myelin-stained transverse sections of sciatic and popliteal nerves; this was taken as evidence that axonal degeneration was not the main lesion. The only average values that clearly differed either individually or on the whole from the average values of the other groups, were those of the 4 paralysed treated animals in group II, which were reduced; Student's *t*-test showed that subgroups A (10 non-paralysed animals) and B (4 paralysed animals) of group II, were significantly different ($P = 0.05$).

Discussion. Histological evidence of reparative changes clearly attributable to vitamin-B_{12} therapy, has not been found in either the central or the peripheral nervous system. Though we have previously found remyelination in treated animals[2], we have now been able to show that remyelination and occasional axonal regeneration can occur in deficient as well as treated monkeys. Some functional improvement was noted in the latter, which may result from arrest of the degenerative process and recovery of suppressed nerve function.

The findings of Oxnard and Smith[1] in a small number of cases that suggested a greater involvement of the distal parts of the nerves of the lower limbs, possibly due to a 'dying back' of the peripheral parts of the axons, has not been confirmed. The distribution pattern of the lesions found in the present study, is consistent with random involvement of Schwann cells rather than selective damage to the distal axons. It was not possible to ascertain whether the occasional nerve fibre that showed axonal (wallerian) degeneration had been affected primarily or had degenerated as a consequence of Schwann-cell disease.

None of our monkeys showed lesions in the gastrointestinal tract such as mucosal atrophy or parasitic infestation to account for the deficiency of vitamin B_{12}. The possibility that other nutritional defects were also present cannot be entirely disregarded though the similarity to human subacute combined degeneration and the functional improvement noted after treatment, strongly suggest that lack of vitamin B_{12} in the animals' diets was the main causal factor[4].

[1] C. E. Oxnard, W. T. Smith and I. Torres N, Acta neurol. latinoam., in press.

[4] We are indebted to the Agricultural Research Council (Grant No. 40319) and to the Ministerio de Sanidad y Asistencia Social through the Universidad de Oriente, Venezuela for generous financial support.

Résumen. Monos 'rhesus' mantenidos en una dieta vegetariana presentaron niveles séricos de vitamina B_{12} reducidos y lesiones en el sistema nervioso central semejantes a las de la edegeneración combinada sub aguda del humano. Los nervios periféricos mostraron desmielinización segmentaria, compatible con un compromiso de las células de Schwann al azar. Usualmente hubo remielinización segmentaria, asociada a veces con degeneración axónica (walleriana). No se encontró evidencia de remielinización o de regeneración axónica que pudiera ser atribuida totalmente al tratamiento con vitamina B_{12}.

I. Tomkin N., W. Th. Smith and Cit. Ormard

Departments of Anatomy and Pathology, University of Birmingham (England), 20 October 1968.

The Role of the Pancreas in Vitamin B_{12} Absorption: Studies of Vitamin B_{12} Absorption in Partially Pancreatectomized Rats

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ABSTRACT The effect of partial pancreatectomy (80–90%) on vitamin B_{12} absorption was studied in the rat. The absorption of 5 ng of ^{57}Co-labeled vitamin B_{12} was significantly reduced from 70 ±2.5% (mean ±SE) in control and sham-operated rats to 32 ±2.6% in partially pancreatectomized rats. Hog pancreatic extract (0.17 g/kg) improved vitamin B_{12} absorption from 30.0 to 61.0% in partially pancreatectomized rats but did not alter vitamin B_{12} absorption in control rats. Chloramphenicol did not enhance vitamin B_{12} absorption in partially pancreatectomized rats with pancreatic extract–improved vitamin B_{12} malabsorption. The partially pancreatectomized rats with pancreatic extract–improved vitamin B_{12} malabsorption were sacrificed and the stomach and small bowel studied in vitro to further define the pathogenesis of the vitamin B_{12} malabsorption. Rat gastric intrinsic factor stimulated vitamin B_{12} uptake by intestinal sacs prepared from partially pancreatectomized rats 3.1-fold. Gastric intrinsic factor prepared from partially pancreatectomized rats was as effective in promoting vitamin B_{12} uptake by rat intestinal sacs as intrinsic factor prepared from control rats. These data indicate that partially pancreatectomized rats develop an abnormality in the absorption of labeled vitamin B_{12} which can be corrected by pancreatic extract. The vitamin B_{12} malabsorption is due to neither an alteration in gastric intrinsic factor activity nor an impairment of the intrinsic factor–vitamin B_{12} receptor in the intestine. It is suggested that in the partially pancreatectomized rats the intrinsic factor–vitamin B_{12} complex exists in a form which is not available for absorption.

INTRODUCTION

Although several patients with pancreatic exocrine insufficiency have been noted to poorly absorb vitamin B_{12} the importance of normal pancreatic function for optimal vitamin B_{12} absorption is generally not appreciated (1–5). Furthermore, the pathogenesis of the observed vitamin B_{12} absorptive defect has not been clarified.

The relationship between the pancreas and the intestinal absorption of vitamin B_{12} was studied by measuring the absorption of this vitamin in rats subjected to partial pancreatectomy. The results demonstrate (*a*) that the partially pancreatectomized rat has a defect in vitamin B_{12} absorption, (*b*) that the administration of hog pancreatic extract restores vitamin B_{12} absorption to normal, (*c*) that intrinsic factor extracted from the gastric mucosa of partially pancreatectomized rats stimulates vitamin B_{12} absorption in vitro, and (*d*) that gastric intrinsic factor stimulates vitamin B_{12} uptake in gut sacs prepared from partially pancreatectomized rats. These data indicate that partially pancreatectomized rats develop a defect in vitamin B_{12} absorption which is due to neither an alteration in gastric intrinsic factor activity nor to an impairment of the intrinsic factor–vitamin B_{12} receptor in the intestine.

METHODS

Animals. Male albino rats,[1] purchased in groups of 12, were delivered to our animal facilities weighing between 75 and 100 g and were allowed ad lib. stand-

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[1] Charles River Breeding Laboratories, Brookline, Mass.

ard lab chow[3] and water. When they reached a weight of approximately 130–150 g rats were selected at random for pancreatic surgery while others served as sham-operated or unoperated controls.

Surgery. Partial pancreatectomies were performed as described by Scow (6). In brief, rats were fasted for 18–20 hr and anesthetized by intraperitoneal pentobarbital (40 mg/kg). The abdomen was entered via a mid-line incision and the pancreas exposed. The rat pancreas consists of three major segments; the gastrosplenic segment comprises 70% of the total pancreatic mass, the duodenal segment nestled in the medial aspect of the duodenal loop contributes about 25%, and the small biliary segment surrounding the bile duct constitutes approximately 5% of the total pancreatic mass. The entire gastrosplenic and a variable portion of the duodenal segment were stripped by rolling small cotton swabs across the parenchyma but the biliary segment was allowed to remain intact; thus 80–90% of the pancrease was extirpated. Sham operations were performed in a similar manner except that the pancreas was manipulated rather than excised. A minimum period of 2 wk elapsed before absorption studies were performed. Operated rats lagged behind the growth rate of control animals for 5–7 days but by the beginning of the second postoperative week the absolute weight and rate of growth were comparable in both groups.

Vitamin B_{12} absorption studies. 1 ml of B_{12}-^{57}Co[4] containing 5 ng of vitamin B_{12} (13–15 μCi/μg) was administered via a gastric tube to rats having access to only water for 12 hr. The rats were placed in restraining cages[5] that allowed for complete separation of urine and feces. 2 hr after gastric intubation the rats were allowed free access to food and water. Stools were collected for 4–5 days at the end of which time less than 1% of the orally administered dose appeared in the stool per day.

Effect of pancreatic extract on vitamin B_{12} absorption. Hog pancreatic extract[6] (0.17 g/kg) was administered via gastric tube immediately after the labeled vitamin B_{12}.

Effect of chloramphenicol on vitamin B_{12} absorption. Chloramphenicol[6] was added to the drinking water for 5 days before and during the 5 days required to complete the vitamin B_{12} absorption studies. Each rat ingested from 50 to 75 mg of chloramphenicol per day.

Vitamin B_{12} uptake by everted rat gut sacs. Rats were sacrificed by decapitation and the small bowel from the ligament of Treitz to the ileocecal valve was perfused *in situ* with 0.9% NaCl at room temperature. The mesentery was removed and the gut was divided into four quarters beginning at the ligament of Treitz. All but the third quarter was discarded. This segment was everted on a metal probe and gut sacs were prepared as previously described (7). The sacs were filled with Krebs-Ringer bicarbonate (8) and incubated separately in Erlenmeyer flasks containing 2 ml Krebs-Ringer bicarbonate with 100 mg/100 ml D-glucose and 1 ng/ml B_{12}-^{57}Co. Rat intrinsic factor (IF) was prepared by scraping the mucosa of the glandular portion of the stomach and homogenizing in saline. 0.1 ml of homogenate (containing 5 mg wet weight of gastric mucosa) was added to alternate flasks. Incubation was carried out in a oscillating water bath for 1 hr at 37°C. The sacs were removed, drained, rinsed briefly in ice-cold saline, weighed, and counted. Results were expressed as nanograms of vitamin B_{12} accumulated by the sac per gram wet weight per hour.

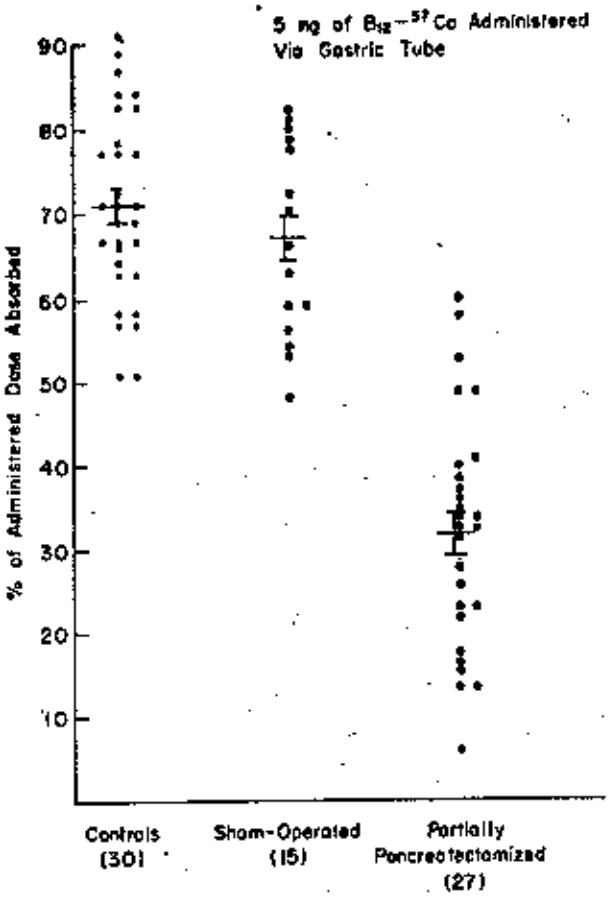

FIGURE 1 Vitamin B_{12} absorption in control, sham-operated, and partially pancreatectomized rats, means ±SE.

Fecal fat analyses. Partially pancreatectomized rats and control rats were placed in metabolic cages for 3–6 days. Quantitative measurement of the food intake and stool output were made. Stool and food were analyzed for fatty acid content by the method of Van de Kamer, Ten Bokkel Huinink, and Weyers et al. (9) and the results expressed as the coefficient of absorption.

Transit time. A dose of 1 ml of a solution containing sodium chromate-^{51} was administered via a gastric tube to fasted rats. The rats were then placed in restraining cages and stool collected at 4-hr intervals. Results were expressed as the cumulative per cent excretion of the administered dose.

Counting procedure. Stools were brought to dry weight by incubating overnight at 95°C and counted with a well-type scintillation crystal with a gamma ray spectrometer and scalar[7] under controlled geometric conditions at window settings appropriate for ^{57}Co or ^{51}Cr.

Statistical analyses were performed using the Student's *t* test.

[3] Wayne Lab-Blox, Allied Mills, Inc., Chicago, Ill.
[4] Squibb Institute for Medical Research, New Brunswick, N. J.
[5] Aerospace Industries, Garnerville, N. Y.
[6] Viokase, VioBin Corp., Monticello, Ill.
[6] Parke, Davis & Co., Detroit, Mich.
[7] Picker Nuclear, White Plains, N. Y.

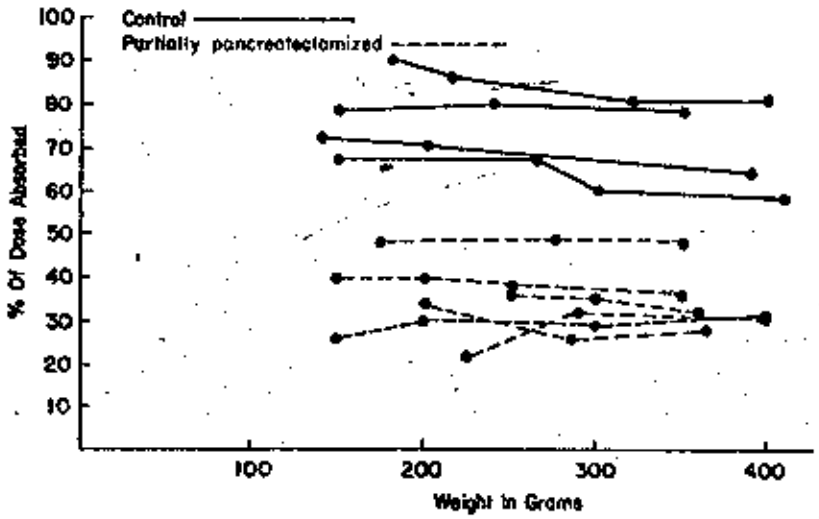

FIGURE 2 Results of repeated vitamin B_{12} fecal excretion tests in control and partially pancreatectomized rats.

RESULTS

Vitamin B_{12} absorption in control, sham-operated, and partially pancreatectomized rats As shown in Fig. 1, 30 control rats absorbed 71.0 ±2.0% (mean ±SEM) and 15 sham-operated rats absorbed 67.2 ±2.9% of the administered vitamin B_{12} ($P > 0.05$). By contrast, 27 partially pancreatectomized rats absorbed 32.6 ±2.6% ($P < 0.01$ compared to both control and sham-operated rats). All control rats and all but one of the 15 sham-operated rats absorbed greater than 50% of the administered dose, whereas 24 of the 27 of the partially pancreatectomized rats absorbed less than 50%.

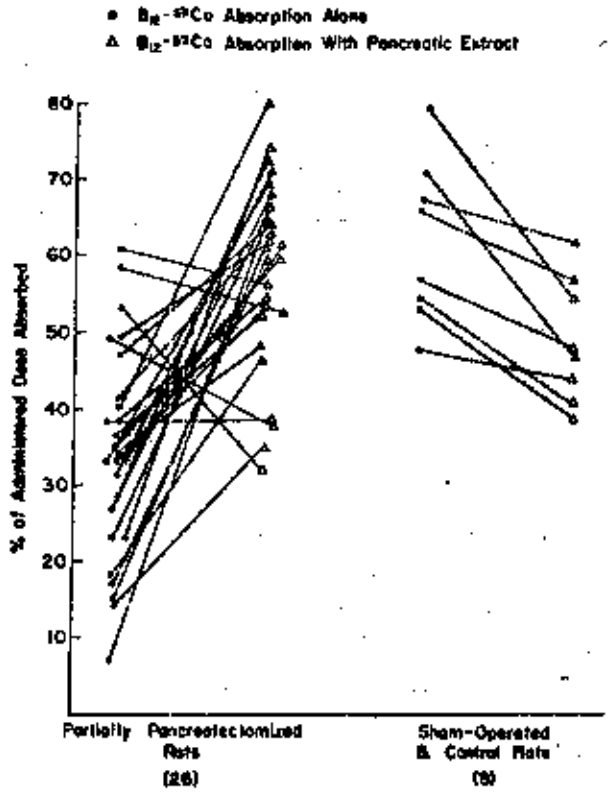

FIGURE 3 Effect of hog pancreatic extract (0.17 g/kg) on vitamin B_{12} absorption in partially pancreatectomized, sham-operated, and control rats.

Results of repeated fecal excretion tests to assess the reproducibility of vitamin B_{12} absorption. Vitamin B_{12} absorption was assessed over a 5 month period during which time body weight increased from 100 to 400 g. As shown in Fig. 2, all four control rats absorbed greater than 50% of the administered dose while vitamin B_{12} absorption was consistently less than 50% in the partially pancreatectomized rats. The ability to absorb vitamin B_{12} in a given rat was quite reproducible during the study period. No value departed by more than 16% from the mean value obtained from averaging all studies performed in a given rat over a 5 month period.

Effect of pancreatic extract on vitamin B_{12} absorption. Hog pancreatic extract (0.17 g/kg body weight) was administered concurrently with labeled vitamin B_{12} to 26 partially pancreatectomized rats. Absorption increased from 33.2 ±2.6% to 57.8 ±2.4% of the administered dose ($P < 0.01$) (Fig. 3). Pancreatic extract increased vitamin B_{12} absorption in 21 of the 26 partially pancreatectomized rats from a mean of 30.0 ±2.4% to 61.0 ±2.3% ($P < 0.01$). Vitamin B_{12} absorption was restudied in 13 partially pancreatectomized rats with vitamin B_{12} malabsorption (29 ± 3.3%) that previously responded to pancreatic extract (62.5 ±3.7%). A value similar to that observed in the initial study period was obtained ([32.4 ±2.7] data not shown). In one rat pan-

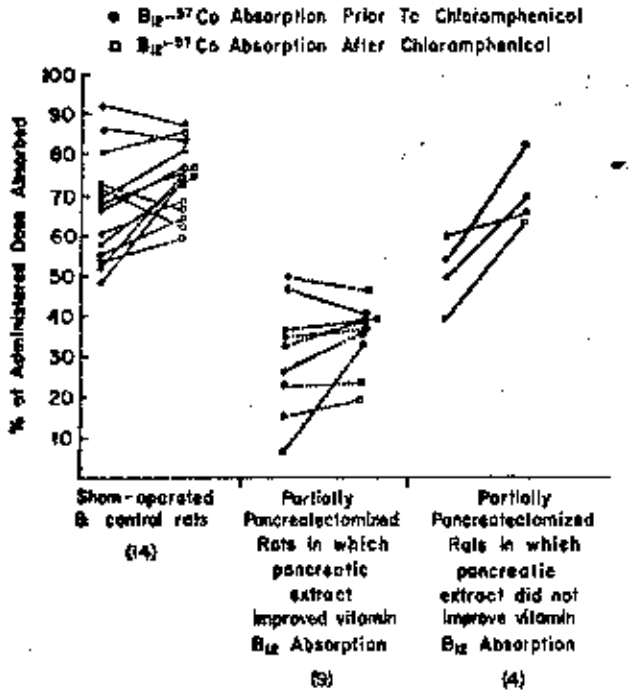

FIGURE 4 Effect of chloramphenicol (50–75 mg/day) on vitamin B_{12} absorption in sham-operated, control, and partially pancreatectomized rats.

creatic extract failed to alter vitamin B_{12} absorption. The four partially pancreatectomized rats that demonstrated the least impairment of vitamin B_{12} absorption after surgery showed a further decrease (19%) in vitamin B_{12} absorption when pancreatic extract was administered. Pancreatic extract administered to eight control and sham-operated rats decreased vitamin B_{12} absorption by 14%.

Effect of chloramphenicol on vitamin B_{12} absorption. The effect of chloramphenicol on vitamin B_{12} absorption was studied in three groups of rats: (*a*) 14 control and sham-operated rats, (*b*) 9 partially pancreatectomized rats in which pancreatic extract had improved vitamin B_{12} absorption 104%, and (*c*) 4 partially pancreatectomized rats in which pancreatic extract failed to improve vitamin B_{12} absorption (Fig. 4). In control and sham-operated rats chloramphenicol failed to significantly ($P > 0.05$) improve vitamin B_{12} absorption (66.1 ±3.5 to 71.8 ±2.4). Similarly, chloramphenicol did not significantly ($P > 0.05$) alter vitamin B_{12} absorption in partially pancreatectomized rats with pancreatic extract improved vitamin B_{12} absorption (30.0 ±4.3% to 34.6 ±2.8%). The four partially pancreatectomized rats that failed to respond to pancreatic extract showed a 38% enhancement of vitamin B_{12} absorption from a mean of 50 to 68.8% of the administered dose.

TABLE I

Fecal Fat Excretion in Control, Sham-Operated, and Partially Pancreatectomized Rats

	No. of rats	Coefficient of absorption*
Control and sham-operated rats	20	89.8 ±0.79‡
Partially pancreatectomized rats	12	85.9 ±1.62‡

* (Fatty acid intake—fatty acid output in stool)/(fatty acid intake) × 100.

‡ Values given are mean ±SE and were not significantly different, $P > 0.05$.

Fecal fat excretion in control and partially pancreatectomized rats. As shown on Table I, when fed a diet containing 5.3% fat, there was no significant difference in the coefficient of absorption between control and pancreatectomized rats.

Stomach to anus transit time in control and partially pancreatectomized rats. As shown in Fig. 5, the rate at which the labeled chromate appeared in the stool was comparable in partially pancreatectomized and control rats.

Ability of intestinal sacs from control and partially pancreatectomized rats to respond to gastric intrinsic factor in vitro. Four partially pancreatectomized rats with impaired vitamin B_{12} absorption (mean of 29% of the administered dose) whose vitamin B_{12} absorption was improved with pancreatic extract (to a mean of 65%) were sacrificed. Everted sacs were prepared from the mid-gut and incubated either with labeled vitamin B_{12} or vitamin B_{12} and rat gastric intrinsic factor (prepared from unoperated rats). As shown in Table II, gastric intrinsic factor stimulated vitamin B_{12} uptake a mean of 3.1-fold as compared to 2.8-fold stimulation with sacs prepared on the same day from control rats of a similar size.

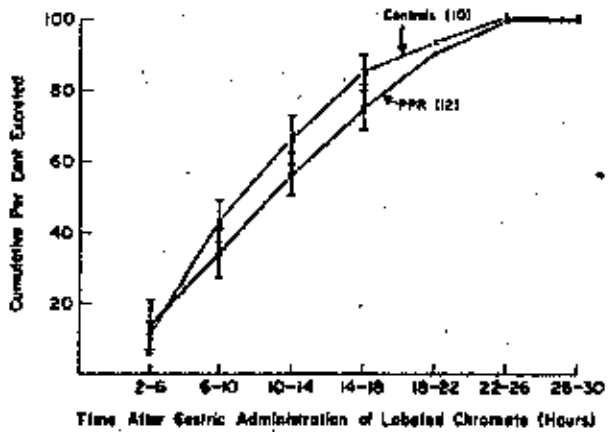

FIGURE 5 The stomach to anus transit time of labeled chromate in 10 control and 12 partially pancreatectomized rats (PPR), means ±SE.

TABLE II

Effect of Rat Gastric Intrinsic Factor Obtained from Control Rats on Vitamin B_{12} Uptake by Intestinal Sacs Prepared from Partially Pancreatectomized Rats

	Weight at time of sacrifice	Vitamin B_{12} uptake		IF-mediated B_{12} uptake
		B_{12}	B_{12} + IF	Non-IF-mediated B_{12} uptake
	g	*mg/g wet weight per hr*		
Partially pancreatectomized rats				
1	385	0.24 (4)	0.88 (4)	3.7
2	365	0.27 (4)	0.67 (5)	2.4
3	555	0.28 (3)	0.83 (4)	3.0
4	405	0.43 (4)	1.5 (3)	3.4
Control rats				
1	345	0.26 (4)	1.00 (4)	3.8
2	445	0.32 (4)	0.82 (5)	2.6
3	365	0.42 (8)	0.82 (6)	2.0

The number in parentheses denotes the number of sacs studied.

Comparison of gastric intrinsic factor prepared from unoperated rats with intrinsic factor prepared from partially pancreatectomized rats. When rat intrinsic factor obtained from gastric homogenates from partially pancreatectomized rats was added to intestinal sacs prepared from control rats, the stimulation of vitamin B_{12} uptake was comparable to the enhancement of uptake obtained with intrinsic factor prepared from control rats (Table III).

DISCUSSION

The present schema for vitamin B_{12} absorption envisages the ingestion of vitamin B_{12} (actually coenzyme B_{12} bound to protein [10–13]), release from its protein bond as a result of proteolytic digestion in an acid medium, binding to intrinsic factor in the stomach or upper portion of the small bowel (14–16), passage down the small bowel, attachment of the IF-B_{12} complex to a specific receptor located in the brush border of the ileal epithelial cell (17–21), and eventual release of the vitamin into the portal circulation unaccompanied by IF (22, 23). In addition a factor elaborated by the pancreas may be an obligatory requirement for optimal vitamin B_{12} absorption. Such a feature has been suggested by the observation that among patients with unexplained vitamin B_{12} malabsorption (measured by the urinary excretion test [24]) were several with pancreatic insufficiency (1, 2). The pathogenesis of the observed vitamin B_{12} absorptive defect has not been clarified, although the improvement in vitamin B_{12} absorption after sodium bicarbonate administration suggested that an increased hydrogen ion concentration in the ileal lumen accounted for the malabsorption (3). However, the improvement in vitamin B_{12} absorption after the administration of pancreatic extract for several days (3) or as a single dose concomitant with the labeled B_{12} (5) cannot be explained on the basis of altered luminal pH. Furthermore, the pH of the ileal contents of subjects with pancreatic insufficiency and vitamin B_{12} malabsorption was similar to that of patients with pancreatic insufficiency and normal vitamin B_{12} absorption and to that of normal volunteers (5). Calcium ions have also been demonstrated to be necessary for IF-mediated vitamin B_{12} uptake in vitro (25, 26). It has been suggested that in severe fat malabsorption large amounts of calcium soaps may form to lower the intraluminal concentration of ionic calcium to levels too low to allow for the vitamin B_{12}–IF complex to attach to the ileal receptors (27–29). However, the total calcium concentration in the ileal aspirates was similar in subjects with pancreatic insufficiency with normal or abnormal vitamin B_{12} absorption or in normal volunteers (5).

The data in this study indicate that rats subjected to 80–90% pancreatectomy absorbed significantly less orally administered labeled vitamin B_{12} compared to control and sham-operated animals. The partially pancreatectomized rats appeared healthy, had a growth rate comparable to control rats (data not shown), and in accord with the results of previous authors showed no evidence of fat malabsorption (30). Since the rate at which a non-absorbable marker (sodium chromate) appeared in the stool after intragastric instillation was similar in partially pancreatectomized rats and control rats, differences in the rate of movement down the small bowel would appear to be an unlikely explanation for the vita-

TABLE III

Comparison of Gastric Intrinsic Factor Obtained from Control and Partially Pancreatectomized Rats on Vitamin B_{12} Uptake by Rat Intestinal Sacs Prepared from Control Rats

Incubation conditions	No. of sacs studied	B_{12} uptake*
		mg/g wet weight per hr
B_{12} alone	24	0.33 ±0.024
B_{12} + intrinsic factor prepared from control rats	13	0.83‡ ±0.047
B_{12} + intrinsic factor prepared from partially pancreatectomized rats	12	0.83‡ ±0.055

* Values given are mean values ±SE.

‡ Values are significantly different from that observed with vitamin B_{12} alone.

min B_{12} malabsorption. It is possible, however, that the measurement of stomach to anus transit time may not adequately reflect the transit time in a localized area of the gastrointestinal tract (31).

To determine whether the observed defect in vitamin B_{12} absorption was related to a specific deficiency of a pancreatic factor or to bacterial overgrowth consequent to small bowel manipulation, the effect of pancreatic extract and a broad spectrum antibiotic (chloramphenicol) on vitamin B_{12} absorption were compared. When pancreatic extract was administered to 26 partially pancreatectomized rats, vitamin B_{12} absorption was noted to increase in 21 (from a mean of 30 to 61% of the administered dose) and remained unchanged or decreased in five rats. Nine randomly selected rats with pancreatic extract–improved vitamin B_{12} absorption were placed on chloramphenicol. Vitamin B_{12} absorption increased slightly (although not significantly at the 5% level). Control and sham-operated rats also exhibited a slight although statistically insignificant increase in vitamin B_{12} absorption when chloramphenicol was added to their drinking water. Improvement in vitamin B_{12} absorption in rats after chloramphenicol administration has been observed by others (32). When chloramphenicol was administered to four partially pancreatectomized rats that failed to show improvement in vitamin B_{12} absorption with pancreatic extract, the absorption of this vitamin improved 38% (from a mean of 50 to 68.8%). The latter value is in the range for vitamin B_{12} absorption observed in unoperated rats. It is of interest that the partially pancreatectomized rats that responded to pancreatic extract had a more severe impairment of vitamin B_{12} absorption (30%) as compared to partially pancreatectomized rats that failed to improve with pancreatic extract (50%). Thus, rats subjected to a 80–90% pancreatectomy consistently absorbed vitamin B_{12} poorly and could be segregated into two distinct groups based on their responses to pancreatic extract and chloramphenicol. One group consisted of a small number of rats that had a modest impairment of vitamin B_{12} absorption. Vitamin B_{12} absorption in this group failed to improve with pancreatic extract but was restored to values observed in control rats after chloramphenicol administration. Presumably, the mechanism of vitamin B_{12} malabsorption in this group was related to bacterial overgrowth secondary to surgery. Most rats subjected to a partial pancreatectomy fell into a second category characterized by a more severe defect in vitamin B_{12} absorption that improved with pancreatic extract but was not altered by the administration of a broad spectrum antibiotic. In these rats vitamin B_{12} malabsorption was apparently secondary to a deficiency of a factor elaborated by the pancreas.

There are a number of possible mechanisms by which a pancreatic factor may influence vitamin B_{12} absorption. Studies of gastric intrinsic factor and intestinal sacs from partially pancreatectomized rats with pancreatic extract–improved vitamin B_{12} malabsorption allowed for further definition of the pathogenesis of the vitamin B_{12} absorptive defect. First, a pancreatic factor may be required to interreact with either the vitamin B_{12} binding site of intrinsic factor or the site on the intrinsic factor molecule that attaches to the receptor in the brush border of the ileal epithelial cell. Evidence against the pancreatic factor acting directly upon intrinsic factor was obtained from the observation that vitamin B_{12} uptake in intestinal sacs prepared from control rats was stimulated by rat gastric intrinsic factor obtained from partially pancreatectomized rats. Second, a pancreatic factor may be required to modify the intestinal receptor in order for it to combine with the intrinsic factor–B_{12} complex or the receptor may be synthesized by the pancreas and secondarily attached to the brush border (33). This also seems unlikely since vitamin B_{12} uptake by intestinal sacs from partially pancreatectomized rats with vitamin B_{12} malabsorption was readily stimulated by intrinsic factor prepared from gastric homogenates of control rats. Third, there is evidence that vitamin B_{12} may form complexes in the gastrointestinal tract (34–37), which may not be readily absorbed. The malabsorption observed in partially pancreatectomized rats may be explained by the failure to maintain the intrinsic factor –B_{12} complex in a form that is available for absorption (37). Alternatively, an inhibitor to intrinsic factor–mediated vitamin B_{12} absorption (38) may be present whose activity is maintained at a minimal level by a normal supply of a component of the pancreatic secretion. In the partially pancreatectomized rats, the activity of this inhibitor may suffice to impair vitamin B_{12} absorption. Finally, the surgical procedure may have altered the enterohepatic circulation of vitamin B_{12} (39) leading to an expansion of the vitamin B_{12} pool in the upper gastrointestinal tract of partially pancreatectomized rats. The orally administered labeled cyanocobalamin would therefore be diluted in a larger pool of nonradioactive vitamin B_{12} in partially pancreatectomized rats as compared to controls and would lead to the observed differences in the absorption of labeled vitamin B_{12}.

These results indicate that partial pancreatectomy in the rat induces a defect in the absorption of labeled vitamin B_{12} which was corrected by the administration of exogenous pancreatic extract. This animal model should prove useful in determining the pathophysiology of the vitamin B_{12} malabsorption (as measured by the urinary excretion test) associated with pancreatic exocrine insufficiency in the human.

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6

SPECIFIC LOCALIZATION OF LABELED HORMONES AND VITAMINS; WHOLE-BODY AUTORADIOGRAPHIC OBSERVATIONS

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In the study of the distribution in the body of a physiological substance such as a hormone or a vitamin, the most interesting question generally is: Does the substance concentrate specifically where it acts or is the distribution pattern mainly determined by other factors such as solubility?

Specific uptake in the sites of action is best studied by using substances with a well-localized physiologic response. Estrogens are especially suitable, as histological investigations concerning cell proliferation rate have indicated targets at the tissue and cellular level. The distribution pattern of estrogens can be compared with that of substances to which they are related in various respects; natural estrogens can be compared with each other, with non-estrogenic steroids, and with non-steroid estrogenic compounds.

Autoradiographic distribution investigations were carried out on C^{14}-estrone and H^3-estradiol (Ullberg and Bengtsson, 1963); C^{14}- and H^3-diethylstilbestrol (Bengtsson and Ullberg, 1963; Bengtsson *et al.*, 1963); C^{14}-labeled vitamin A (Ullberg, Appelgren, and Hammarström, unpublished); Co^{58}-labeled vitamin B_{12} (Ullberg, Kristoffersson, and Hanngren, unpublished); and C^{14}-labeled vitamin E (Appelgren, Hammarström, and Ullberg, unpublished).

Whole-body autoradiography has been the basic method applied. Complementary observations have been made in some cases by detailed autoradiography and by chromatographic separation of labeled metabolites.

One of the main advantages of the whole-body autoradiographic method is that it favors the disclosure of unexpected findings as compared with most other methods which pre-select organs for examination.

METHODS

SUBSTANCES

Estrogens:
1. Estrone-16-C^{14} (Radiochemical Center [RCC], Amersham, England), specific activity 12 mc per mmole.
2. Estra-3:17 (β) diol-H^3 (RCC), specific activity 100 mc per mmole.
3. Diethylstilbestrol (monoethyl-1-C^{14}) (RCC), specific activity 12.6 mc per mmole.
4. Diethylstilbestrol-H^3 (RCC), specific activity 295 mc per mmole.
5. The macromolecular compound, polydiethylstilbestrol phosphate-C^{14} (PSP), was prepared from monoethyl-1-C^{14} diethylstilbestrol (RCC), specific activity 10 μc per mg.

Vitamin A: Vitamin A-6,7-C^{14}-alcohol from vitamin A acid (Hoffman la Roche), specific activity 7.66 μc per mg.

Vitamin B_{12}: Cyanocobalamine labeled with Co^{58} (Duphar Laboratory, Holland), specific activity 170 mc per mg.

Vitamin E: DL-α-Tocopherol-acetate-8-methyl-C^{14}, specific activity 4.3 μc per mg.

ADMINISTRATION, DOSE, AND TIME INTERVALS

The labeled estrogens (except PSP) were dissolved in peanut oil and 0.2 mg substance per mouse injected subcutaneously. PSP was dissolved in water and 0.4 mg was injected intravenously. The vitamins A and E were dissolved in peanut oil and 0.2 mg was given orally or subcutaneously. The vitamin B_{12} was dissolved in saline and 0.056 μg was injected intravenously. The time intervals between administration and sacrifice of the animals for whole-body autoradiography were generally 5 minutes, 20 minutes, 1 hour, 4 hours, 24 hours, and 4 days; for vitamin B_{12} also 16 days and 32 days; for vitamins A and E, only 24 hours.

WHOLE-BODY AUTORADIOGRAPHIC TECHNIQUE

A number of animals were given a single dose each of a labeled preparation and at various intervals killed by immersion in hexane at the temperature of solid CO_2. They were then mounted in carboxy-methyl-cellulose ice and a number of sagittal sections (generally 20 μ thick) were taken at various levels at $-10°$ C. The sections were frozen-dried and autoradiographic exposure was made by apposition against photographic film (X-ray film for the C^{14}-labeled compounds, and Ilford G5 nuclear emulsion for the H^3-labeled compounds).

A common exposure time was 3 weeks. For a detailed description of the technique see Ullberg (1954, 1958, 1962).

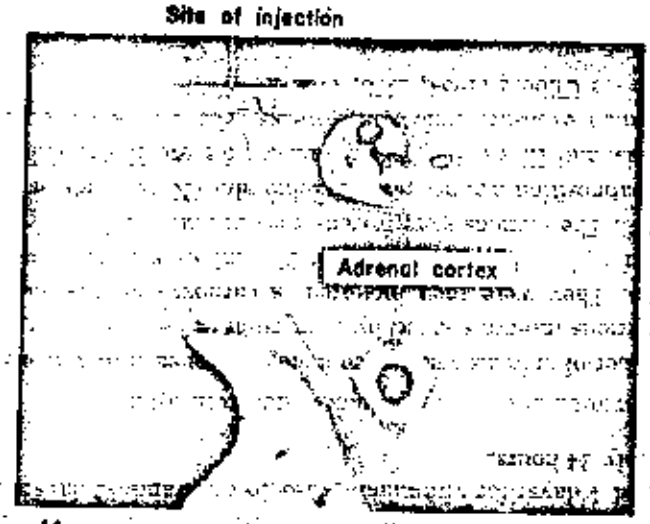

PLATE 1 *Autoradiogram showing distribution of C^{14}-estrone in male mouse 4 hours after subcutaneous injection. The white areas correspond to radioactivity. Note the high concentration in adrenal cortex and liver. Inset shows the adrenal and surrounding tissue enlarged.*

DETAILED AUTORADIOGRAPHY

Fresh-frozen tissue specimens or frozen-dried and paraffin-embedded specimens were used, and 5 μ thick sections were dry-mounted on nuclear plates (Ilford G5) (emulsion thickness 5 μ) or on Kodak AR 10 stripping film previously stretched on chrome-alum treated glass slides. The adhesion between emulsion and section can be improved by various agents (Hammarström, Appelgren, and Ullberg, in press). For details see André (1956).

RESULTS AND DISCUSSION

ESTROGENS

After injection with labeled estrogens, the radioactivity was concentrated in a number of tissues (endometrium, vaginal mucosa, and granulosa layer of ovarian follicles) where its localization seemed to be related to the physiological role of the estrogens. In male animals a concentration was observed in the interstitial tissue of the testis. All animals demonstrated a slight concentration in the pituitary and a very pronounced accumulation in the adrenal cortex (Plate 1).

The subcutaneously administered radioactive substance reached a barely detectable concentration in the blood stream, but the "target organs" showed higher activity than the surrounding tissues as early as 5 minutes after injection (Plate 2). The sites of accumulation then showed an increased level over other tissues during the whole course of absorption and excretion, but no appreciable specific retention of the accumulated activity was apparent.

The areas of specific uptake seemed to be qualitatively the same for the two natural hormones studied (estrone and estradiol) and also for the synthetic one, diethylstilbestrol, with the penetration into and distribution within the fetus being the one exception. The distribution pictures suggested an active transfer of the natural estrogens and/or their metabolites into the fetus but a partial placental block for C^{14}-diethylstilbestrol.

It should be noted that a strong accumulation was apparent in the endometrium while no appreciable accumulation could be observed in the muscle layer of the uterus (Plate 3). In the oviduct, however, the level of radioactivity of the muscle layer was higher than that of the mucosa (Bengtsson and Ullberg, unpublished).

Microautoradiograms from non-pregnant mice indicated that the activity of H^3-estradiol was located mainly over the cell nuclei of the endometrium. This suggests that the cell proliferation and carcinogenic effect of estrogens

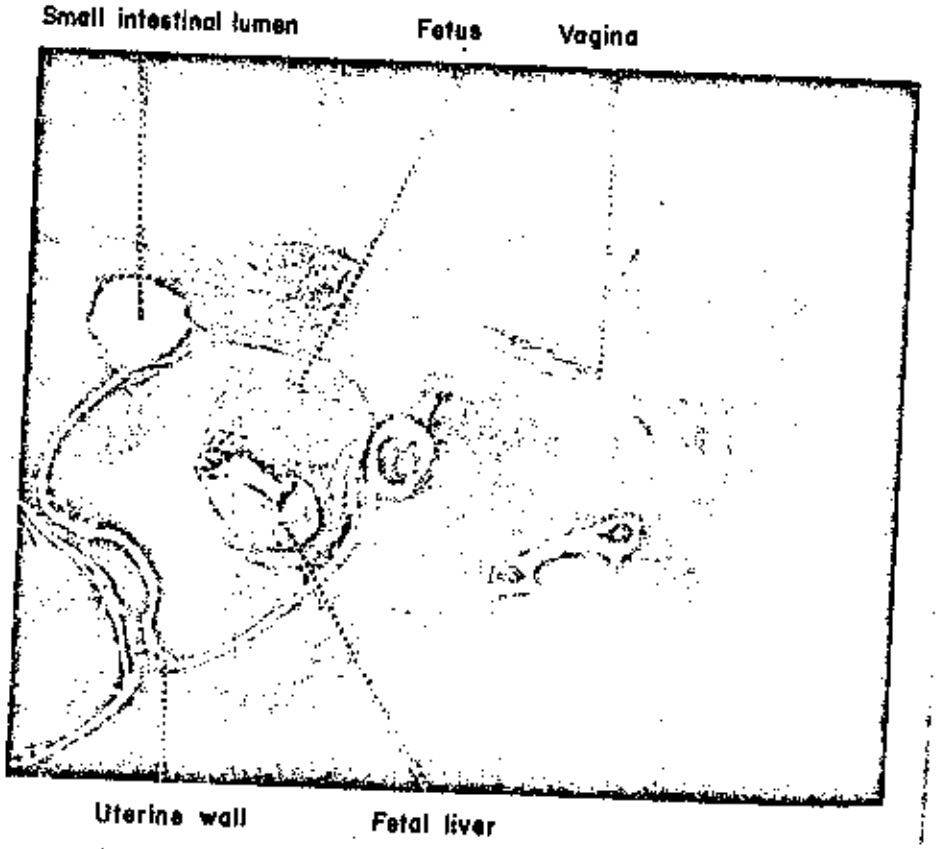

PLATE 2 *A detail of a whole-body autoradiogram from a pregnant mouse 4 hours after subcutaneous injection of C^{14}-diethylstilbestrol. Note high activity in uterine wall, vaginal mucosa, and secretion.*

PLATE 3 *Detailed autoradiogram from uterus of mouse 4 hours after subcutaneous injection of H^3-estradiol. Note high uptake* (black) *in endometrial cells.*

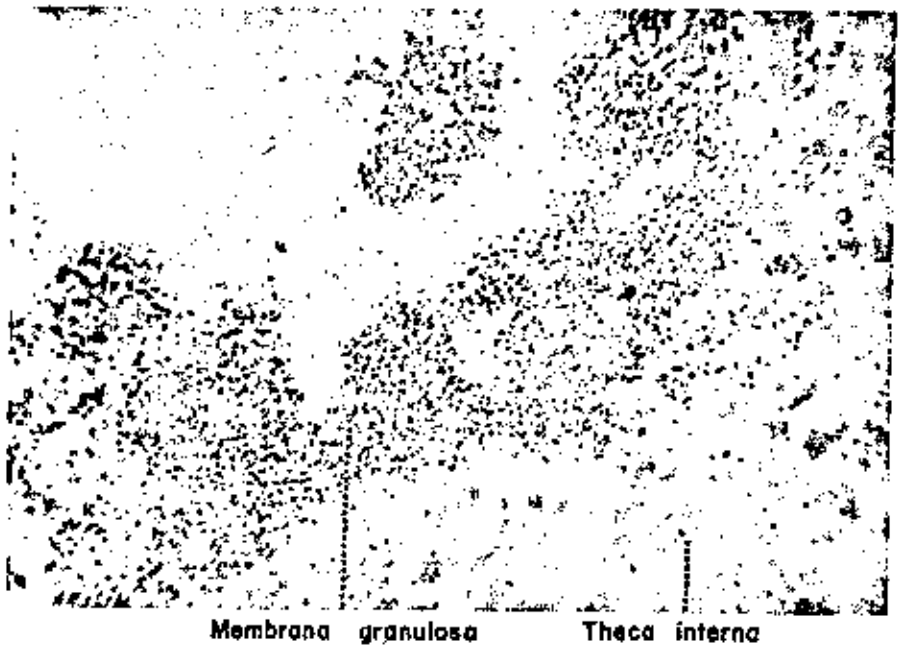

PLATE 4 *Same animals as in Plate 3. Note high uptake of H^3-estradiol in granulosa layer of large ovarian follicle.*

may be caused by a direct action on the cell nuclei. In studies on the ovary, de Wit (1953) and Payne and Runser (1958) observed that the administration of estrogens caused proliferation of the granulosa cells of the ovarian follicles, which indicated the granulosa as a target tissue (Plate 4). However, the theca layer which, according to histochemical investigations (Dempsey and Basset, 1943) is likely to be a main synthesis site of estrogens, showed no uptake of the injected labeled hormone.

The uptake of natural estrogens in the adrenal cortex could indicate that the adrenal cortex is a general steroid trap. However, whole-body autoradiographic investigations with C^{14}-cortisone (Hanngren, Hansson, Sjöstrand, and Ullberg, 1964) showed a total absence of accumulation of radioactivity from this steroid in the adrenal cortex. This also shows that exogenous cortisone is not taken up in tissue where it is formed. The observed accumulation of estrogens is remarkable as it is generally believed (Grant, 1962) that estrogens are synthesized in the adrenal cortex, thus implying that they are specially taken up at a site where they are produced.

The demonstrated specific uptake in the adrenal cortex of injected estrogens also implies specific adrenal uptake of estrogens formed endogenously, for instance, in the gonads. Thus, a demonstration by chemical or biological assay of the presence of estrogens in the adrenal gland is obviously no evidence for estrogen formation within the organ. According to Grant (1962) the formation of estrogens from any precursor by normal adrenal tissue *in vitro* has not been demonstrated. The ability of the normal adrenal gland to produce estrogens thus seems doubtful.

After intravenous administration of a macromolecular long-acting stilbestrol compound (C^{14}-polydiethylstilbestrol phosphate) the polymer was apparently taken up by the reticuloendothelial cells of liver, spleen, bone marrow, and lung. The uptake in these tissues showed a dotted, or scattered pattern. Apparently the monomer was slowly released from the reticuloendothelial depot and transferred to the target organs (such as endometrium and adrenal cortex). The activity in the target organs stood out more clearly against the surrounding tissues than was the case for the diethylstilbestrol autoradiograms which may be due to the slow release from the depots.

VITAMIN A

The radioactivity from labeled vitamin A was concentrated in the retina of the eye. Plate 3 shows an autoradiogram from the head, neck, and shoulder region of a mouse which 24 hours previously had been injected subcutaneously with vitamin A-C^{14}. Microautoradiography of the eye showed the radioactivity in the layer of rods and cones. This discovery is in agreement with the findings of Wald (1960) that vitamin A as the aldehyde is combined with the protein, opsin, to form the light sensitive material in the outer segment of the receptor layer.

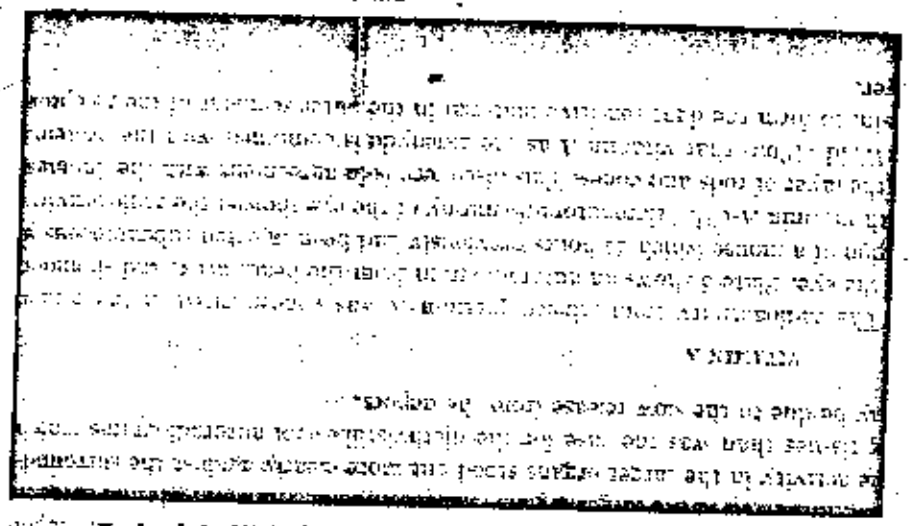

PLATE 5 *Head and shoulder region of whole-body autoradiogram of a mouse 24 hours after intramuscular injection of C^{14}-labeled vitamin A. Note selective uptake in retina of the eye.*

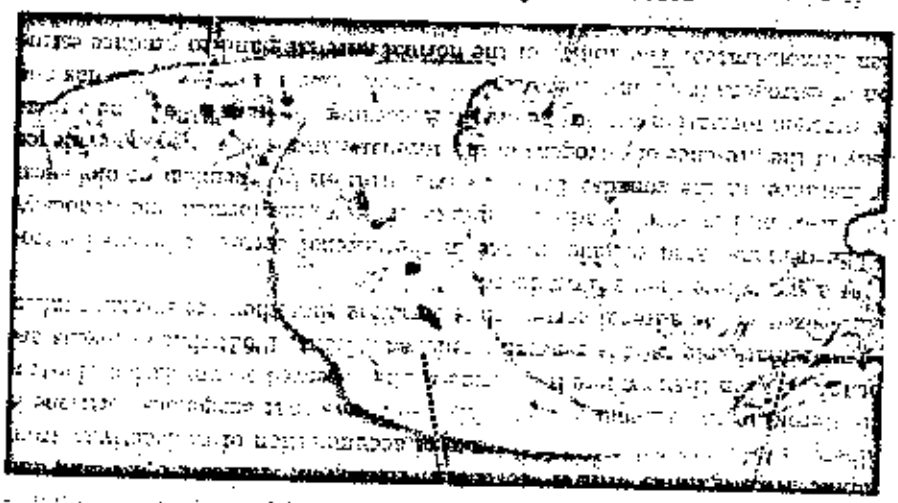

PLATE 6 *Detail of whole-body autoradiogram of mouse 24 hours after subcutaneous injection of C^{14}-labeled vitamin A. Note high uptake in lung, liver, and the esophageal portion of the stomach which is covered with squamous epithelium. Also note the low activity in the glandular part of the stomach*

Autoradiograms from full term pregnant females show that the retinas of the fetuses do not take up the vitamin although the fetal liver shows a slight accumulation, and incorporation of vitamin A into the retina seems to begin after birth.

If we look at the abdominal region of a vitamin A-injected mouse, uptake is seen in the lung, liver, and part of the gastric mucosa (Plate 6). The other white areas seen in the picture represent activity excreted into the intestinal contents.

In the stomach, marked uptake is observed in the esophageal portion of the gastric mucosa which, in mice, consists of squamous epithelium. The glandular part does not show any noticeable uptake. Most other tissues have very low activity.

VITAMIN B_{12}

Vitamin B_{12} has been found to have a unique distribution pattern. In pregnant mice a great portion of an intravenously injected dose is rapidly taken up into the placenta (Plate 7). After a latency of about an hour the activity is then slowly transferred from the placenta to the fetus (Plate 8). After 24 hours the activity in the fetuses is so much higher than that of the mother that only the fetuses appear on an autoradiogram of a pregnant mouse if the picture is not tremendously over-exposed with respect to the fetuses.

Quantitative determinations show that the relative fetal uptake is dose dependent. With the smallest dose, a fetus to mother ratio of 130 (per gram tissue) was seen.

Strong accumulation was also observed in a fast-growing sarcoma transplanted to the neck region of a mouse (Plate 9). This dramatic uptake was not observed in other more slowly growing tumors. The tendency to accumulate in fetuses and tumors indicates some relation of vitamin B_{12} to cell growth.

The distribution of Co^{58}-vitamin B_{12} within each fetus is very similar to that found in adult animals. Strong accumulation is found in all endocrine organs except the adrenal medulla. Other tissues with high uptake are the renal cortex and a restricted area of the brain stem.

The concentration of vitamin B_{12} in bone marrow is rather low, which is remarkable considering the rapid cell proliferation in this site and the specific role of vitamin B_{12} in the formation of red blood cells observed in many species. One possible explanation for this might be that vitamin B_{12} is not acting on the bone marrow directly but via its influence on folic acid.

The distribution in an adult animal is very similar both 1 hour and 1 month after injection, with the exception of most parts of the brain. The B_{12} molecule has been shown to be very stable in the body, the main part of the radioactive substance representing the intact molecule 2 weeks after injection (Neujahr and Ullberg, unpublished).

PLATE 7 *Autoradiogram of pregnant mouse 15 minutes after intravenous injection of Co^{58}-labeled vitamin B_{12}. Note very high uptake in placenta. No radioactivity is yet appearing in fetuses.*

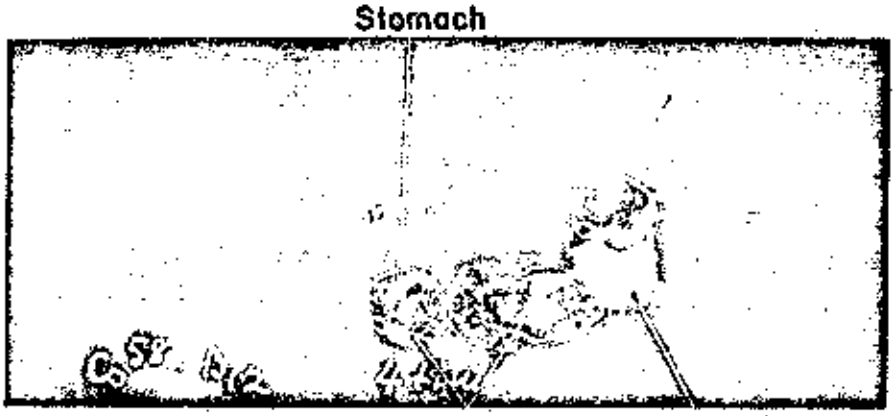

PLATE 8 *Autoradiogram of Co^{58}-labeled vitamin B_{12} in pregnant mouse 4 hours after intravenous injection. Note very high concentration in placenta and that the concentration in the fetal tissues has passed that of the maternal. The radiocobalt concentration is high in the fetal blood but insignificant in maternal blood.*

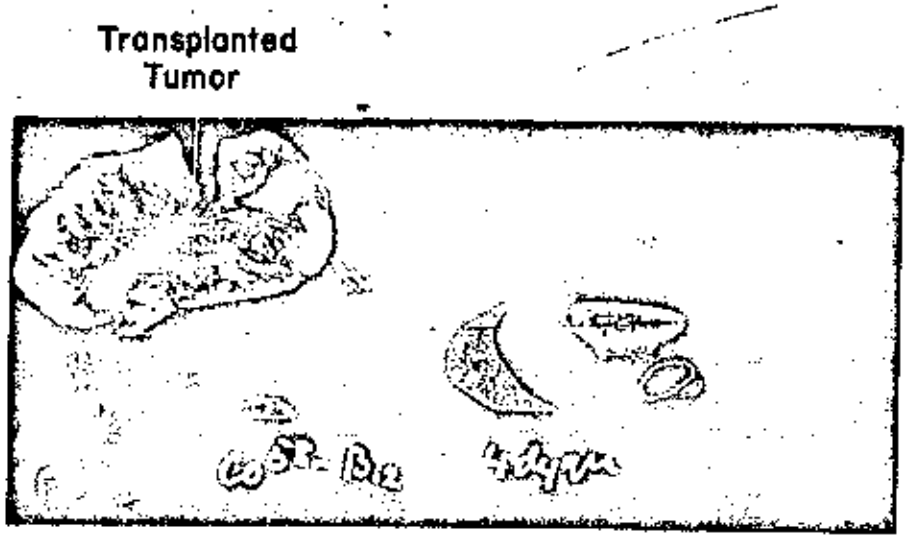

PLATE 9 *Autoradiogram showing high uptake of Co^{60}-labeled vitamin B_{12} 4 days after intravenous injection in a mouse carrying a fast-growing sarcoma transplanted subcutaneously into the neck region.*

VITAMIN E

In small rodents the lack of vitamin E does not generally prevent fertilization but frequently causes death and absorption of the fetuses. According to the autoradiograms (Plate 10), the penetration of C^{14}-labeled vitamin E (and its possible labeled metabolites) to the fetus is almost totally prevented. It therefore seems as if the effect on reproduction cannot be the result of a direct action on the fetuses. A distinct accumulation is, however, seen in the follicular walls and the corpora lutea of the ovary. C^{14}-Labeled vitamin E also accumulates very markedly in bone marrow and in the red pulp of the spleen.

The natural estrogens were concentrated in areas where a physiological response has been shown. Their distribution pattern was very similar to that of C^{14}-diethylstilbestrol. The only organ in the body which was able to distinguish the synthetic from the natural estrogens seems to be the placenta.

Presumably the action of steroid as well as non-steroid estrogenic compounds in the target tissues is connected with a specific uptake on receptor sites.

The tendency of the radioactivity to localize was evident as early as 5 minutes after injection, and the areas of localization appeared to be unchanged at later times. It is notable also that after subcutaneous application, accumulation appeared in target organs without any significant blood concentrations being discernible. No specific retention was apparent in target organs, but with time the relative concentration in excretory organs increased. Earlier data (Twombly, 1951; Valcourt *et al.*, 1955; Ringler, 1957; Sandberg *et al.*, 1959) obtained with impulse counting methods have indicated a concentration

of estrogens only in excretory organs. Autoradiography gives more detailed information; however, it would also appear that earlier investigators waited too long after injection to see the peak concentrations in the target organs.

The experiments with the fat soluble substances showed that fat solubility itself did not influence the distribution pattern to any great extent. It is true that both estrogens and vitamins A and E showed a slight tendency to accumulate in body fat, but each compound also showed its own characteristic distribution pattern.

With respect to one of the best known of all vitamin functions, that of vitamin A in the eye, the labeled compound was taken up predominantly in the expected site of action. Labeled preparations will certainly be of great help in further studies of the role of vitamin A in the retina. Additional experiments are needed to show whether the surprisingly strong localization in the lung indicates a specific function in this organ.

The accumulation of vitamin B_{12} in fetuses and tumors indicates a relation to cell division or protein synthesis. It seems, however, that the specific uptake in endocrine organs must have another explanation.

Certain results obtained with the vitamins are somewhat confusing. Although vitamin A localized, as expected, in the retina, vitamin B_{12} failed to localize specifically in bone marrow. However, vitamin E did accumulate in the marrow. It seems worthwhile to look into these problems further, especially with regard to the possible physiological and pharmacological role of vitamins at unexpected loci where they have been shown to accumulate.

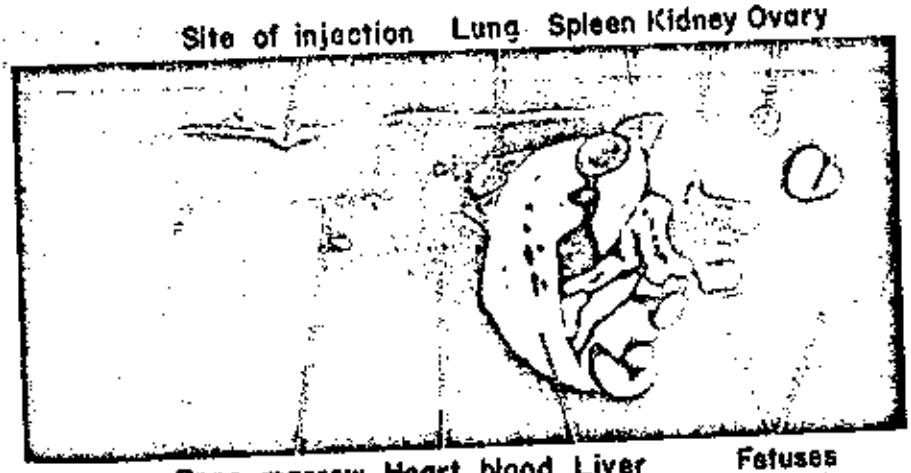

PLATE 10 *Whole-body autoradiogram of mouse 24 hours after subcutaneous injection of C^{14}-labeled vitamin E. No activity can be seen in fetuses. High uptake is seen in liver, follicular walls of ovary, bone marrow, and red pulp of spleen.*

SUMMARY

The distribution pattern of a number of labeled hormones and vitamins has been investigated in mice by whole-body autoradiography complemented by detailed autoradiography of selected organs. The pattern after injection of labeled diethylstilbestrol was almost identical with the pattern after injection of the labeled natural hormones, estrone and estradiol. The radioactivity was taken up specifically in a number of probable target tissues, the endometrium, the vaginal mucosa, the granulosa layer of ovarian follicles, and also in the adrenal cortex.

Injected C^{14}-labeled vitamin A was evenly distributed except for a distinct localization in a few tissues: the retina, liver, and lung. No uptake was found in fetal eyes. The Co^{58}-labeled vitamin B_{12} was highly concentrated in fetuses and in a fast-growing tumor. It also localized in most endocrine glands, but no significant localization appeared in bone marrow. C^{14}-Labeled vitamin E did not pass into the fetus. Localization was found in ovarian follicle walls, corpus luteum, and in bone marrow.

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PLACENTAL PASSAGE AND FETAL ACCUMULATION OF LABELLED VITAMIN B_{12} IN THE MOUSE

BY

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Vitamin B_{12} is mainly known as a therapeutic agent against pernicious anemia in man, but it is also of importance for basic metabolic processes and is necessary for normal growth and reproduction in animals and some microorganisms.

The metabolic mode of action of vitamin B_{12} is not clear, even if several enzymatic *in vitro* reactions involving the vitamin has been elucidated during recent years. Vitamin B_{12}-coenzymes are believed to participate in certain isomerisation reactions (11) and in methyl transfer to homocysteine (7, 10). The vitamin is also claimed to be involved in DNA synthesis (2, 3).

Vitamin B_{12} has many unusual properties. It is active in extremely low concentration; some authors have claimed that it is the most potent of all biocatalysts. Another agent which is also active against megaloblastic anemias, folic acid, is required in about 1000 times higher concentrations. B_{12} is the only vitamin which is synthesized exclusively by microorganisms. It is not present in significant amounts in green plants. Non-carnivorous animals, as a rule, get their supply through microbiological synthesis in their alimentary canal.

The mechanism of intestinal absorption of vitamin B_{12} is also unusual in requiring the presence of a specific substance, the intrinsic factor, which is formed in the gastric mucosa, apparently with the only purpose of facilitating the passage of vitamin B_{12} from the intestinal lumen to the blood.

Investigations of the distribution of vitamin B_{12} in the body have

been made by surface scintillation or by measuring whole separate organs (8, 1[illegible], 20, 23, 24, 25). In the present investigation the distribution in pregnant mice of radiocobalt-labelled vitamin B_{12} (cyanocobalamin) has been studied using whole body autoradiography. This has permitted the simultaneous comparison of the concentrations in the maternal tissues, the placenta and the fetal tissues at various times after administration.

The unusual pattern in the transfer of vitamin B_{12} from mother to fetus which appeared in the autoradiographic work initiated further studies with scintillation counting using a double detector arrangement. The influence of dose and time on the placental transfer has thus been further elucidated.

Materials and Methods

White mice of the American strain N.M.R.I. were used. Diet: The mice were fed mouse pellets and water *ad. lib.* The pellets were supplied by AB Ewos, Södertälje, Sweden, and were composed of: wheypowder 5 %, expanded cereals 71 %, soybean oil meal [illegible] %, fish meal, defatted, 8 %, soybean oil 2 %, yeast 2 %, lime stone 1 %, vitamin concentrate 1 %, trace elements 1 %. Average analysis dry matter: crude protein 20 %, crude fat 4 %, ash 5 % (Ca 0.8 %, P 0.6 %), fiber 3 %, N free extracts 68 %. Water content [illegible] %.

Vitamins added (per 100 grams): vitamin A 360 IU, vitamin D 36 IU, vitamin E 4.5 IU, thiamine 150 micrograms, riboflavin 300 micrograms, pyridoxine 500 micrograms, pantothenic acid 500 micrograms, niacin 1 milligram, cholin chloride 0.1 gram.

Trace elements added (per cent): Fe 0.0216, Cu 0.00017, Co 0.000017, Mn 0.0016, I 0.00006, Zn 0.01[illegible].

The only source of vitamin B_{12} in the diet was yeast, and it contained 50 µg vitamin B_{12} per kg.

Autoradiography.

A series of five pregnant mice were injected with Co^{60}-labelled vitamin B_{12} (cyanocobalamin) with a specific activity of [illegible] mC/mg (Merck). The dose was 0.18 µg/g body weight, corresponding to about 5 µg of B_{12} per animal. As this dose strongly exceeds the physiological need, Co^{57}-labelled vitamin B_{12} (cyanocobalamin) with the remarkably high specific activity of [illegible] mC/mg (obtained from Philips-Duphar, Holland) has been used for [illegible] animals. Every animal has received 0.19 · 10⁻³ µg/g body weight of the Co^{57} vitamin B_{12} corresponding to about 5.7 · 10⁻³ µg B_{12} per mouse. In all [illegible] dose has been given intravenously in a tail vein. The volume injected was [illegible] for Co^{60}-B_{12} and [illegible] for Co^{57}-B_{12}.

The [illegible] days before the expected day of parturition. At different [illegible] after the injection they were anaesthetized and immersed into a mixture of solid carbon dioxide and hexane ([illegible] 75° C). The times between injection and killing [illegible] 4 hours, 24 hours, 4 days, and 20 days and for [illegible] 24 hours, [illegible] days, 16 days, and 32 days. The mice [illegible] vitamin B_{12} 12 days before mating. [illegible] pregnancy the above-mentioned series were

implemented with two mice which were injected with Co^{58}-vitamin B_{12} 9 days after mating and were killed 24 hours later.

The autoradiographic method has been described elsewhere (26, 27). Sagittal 20 μ sections through the whole frozen animals were cut at different levels and dried at −10° C. The films used were Gevaert's Structuris X-ray film and Ilford's G 5 nuclear plates. The exposure time varied between 10 and 60 days.

Scintillation counting.

The apparatus used for the quantitative measurements consisted of two flat sodium iodide crystals (2" × 2"). These were electronically coupled for summation of their individual counts. The specimen was placed equidistant from the two detectors, which faced each other at a distance of 10 cm. This arrangement permitted the measurement of the low activities that were utilized.

Co^{58}-vitamin B_{12} was injected intravenously in a tail vein to pregnant mice under varying conditions. The doses and intervals used will be given in more detail along with the result report. The animal material consisted of 62 female N.M.R.I. mice, 57 of which were pregnant and 5 non-pregnant. The radioactivity of the living animals was determined immediately after the injection and at various intervals. After the animals were killed the fetuses, placentae and remaining carcasses were also counted in the double detector.

To check the reliability of the double detector arrangement the fetuses, placentae and carcasses from four animals, injected with 0.08 μg Co^{58}-B_{12}, were digested in conc. HNO_3, and aliquotes were measured in a well crystal. The difference between the results using the two methods was 1–3 %.

Results

General findings (Scintillation counting + autoradiography).

After the intravenous injection, vitamin B_{12} activity disappeared rapidly from the blood and was taken up by the placentae and by

Table I

Retention (in per cent of injected dose) after 24 hours and relation fetus to mother of the retained radioactivity following injection with various doses of Co^{58}-vitamin B_{12}

Dose in μg	Retention after 24 hours	Activity per g tissue Fetuses:mother
0.02	45.0	140.0
0.04	36.0	11.5
0.22	29.1	10.4
0.50	19.7	10.1
2.02	11.5	6.4
2.50	11.3	2.1
12.50	14.3	2.1
20.02	14.0	
200.02	3.7	1.2

maternal tissues. After 15 minutes (FIG. 3) the concentration in the maternal blood was lower than in most tissues and after 4 hours (FIG. 5a) it was hardly discernible. When high doses were given renal excretion contributed significantly to the clearance of the blood, especially during the first few hours after injection (TABLE II). After administration of

TABLE II

Retention during the first 8 hours after injection with Co^{58}-vitamin B_{12}

	Dose in μg	Retention in per cent after 1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h
Mouse I	0.50	74.2	31.5	22.4	20.0	20.2	19.4	19.0	19.1
Mouse II	2.50	43.0	29.0	21.6	9.6	9.1	8.6	8.5	8.2

low doses, however, most of the radioactivity was retained in the body for a period of 6 days (TABLE III).

The uptake in the placentae was by far the highest of all tissues. Accumulation was also observed in different maternal tissues including certain endocrine organs, the renal cortex, the liver and the gastric mucosa.

TABLE III

Retention of Co^{58}-B_{12} in pregnant and non-pregnant mice, studied from 24 hours until 6 days. Dose: 0.05 μg per mouse

Retention after	Pregnant	Animal number	Non-pregnant	Animal number
24 hours	90.0 ± 0.77	13	83.3 ± 0.96	5
2 days	84.8 ± 0.25	13	79.5 ± 1.90	5
3 days	83.6 ± 0.39	13	77.1 ± 1.18	5
4 days	82.7 ± 0.53	13	73.6 ± 1.58	5
6 days	80.5 ± 0.35	13	70.5 ± 1.96	5

There was a slow transportation of radioactive substance from the placentae to the fetuses. After 24 hours the fetal concentration had reached its peak. The fetal tissues reached much higher concentrations

than the maternal tissues. The fetal uptake was dose dependent. With low doses the fetuses showed 40 to 130 times higher concentrations per gram tissue than the maternal bodies (TABLE I). The distribution pattern of each fetus was very similar to that of the mother. The activity was not collected in a depot organ such as the liver but almost every fetal tissue showed higher concentration than the corresponding maternal. In animals killed a long time after injection (16 to 32 days) there was some redistribution of the radioactive substance and a relatively lower fetal concentration, apparently due to dilution by fetal growth (FIG. 8).

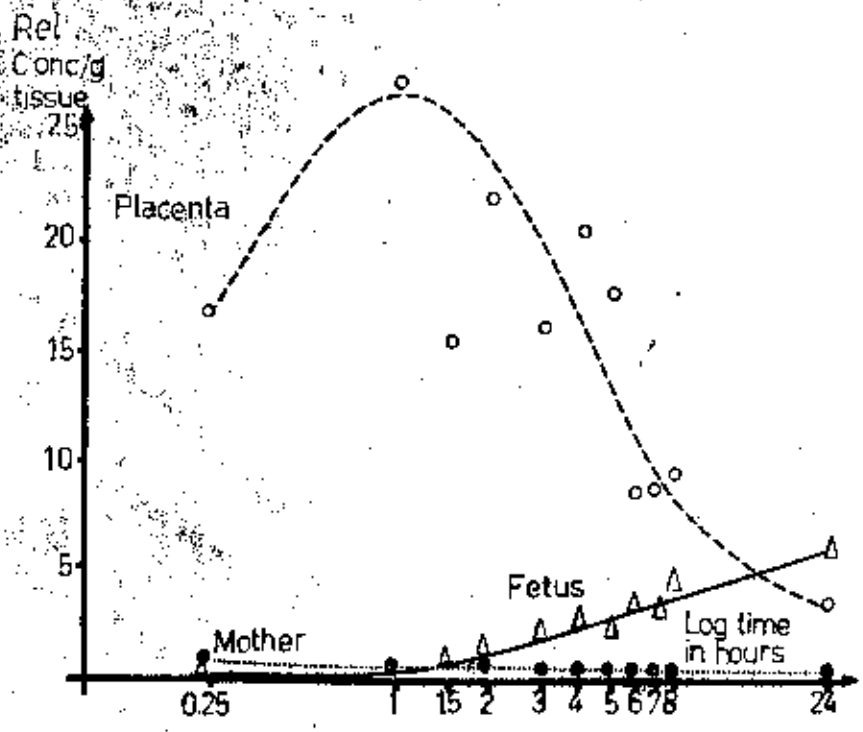

FIG. 1

Relative concentrations of radioactive substance per gram tissue of placentae, fetuses and remaining carcass of the dam studied 15 minutes to 24 hours after injection of Co^{58}-B_{12}. The concentrations are put in relation to the mean concentration of a pregnant mouse, immediately after injection of 0.05 µg radio-B_{12}. Note the rapid accumulation in the placentae and the slow accumulation in the fetuses. Each value indicates the mean from 3 mice.

Scintillation counting.

The effect of the dose size on the retention of radioactivity 24 hours after the intravenous injection of Co^{58}-labelled vitamin B_{12} to full

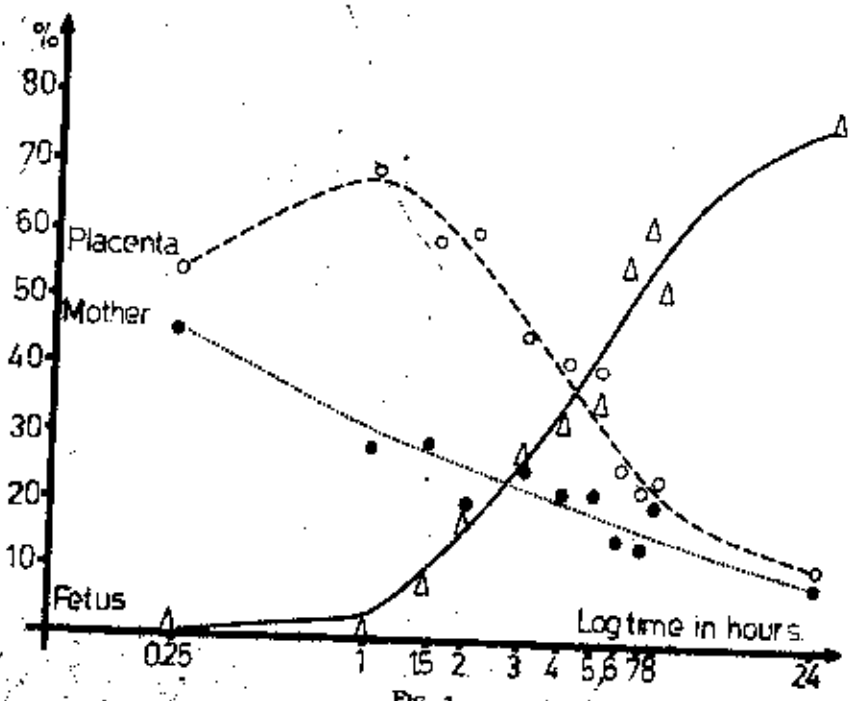

FIG. 2

Content of radioactive substance in the collected placentae, fetuses and remaining carcass in per cent of the whole retained dose 15 minutes to 24 hours after injection with Co^{58}-B_{12}. Dose: 0.05 μg. Most of the labelled vitamin B_{12} is during the first hours located in the placentae and later on in the fetuses. Each value indicates the mean from 3 mice.

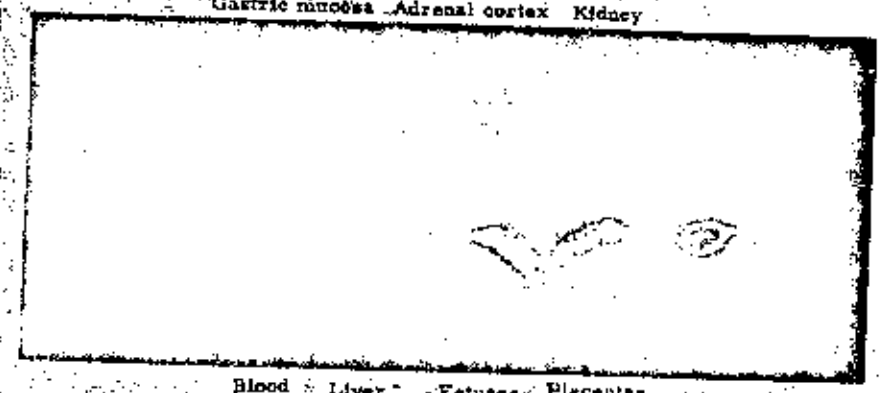

FIG. 3

Autoradiogram of Co^{58}-labelled vitamin B_{12} in a pregnant mouse 15 minutes after intravenous injection. The radioactivity has not yet reached the fetuses but the concentration in the placentae is by far higher than in any other tissue. Several organs such as the kidney, gastric mucosa and adrenal cortex

FIG. 4a

Autoradiogram of Co^{58}-vitamin B_{12} in a pregnant mouse 1 hour after intravenous injection. Radioactivity appears in one of two visible fetuses. In the mother the blood concentration is low. Accumulation is seen in hypophysis, thyroid, choroid plexus, brain stem area and liver.

term pregnant mice can be studied in Table I. When the low dose of 0.02 micrograms per mouse was given, only 5 per cent of the radioactive substance was excreted. With increasing doses the percentage excretion went up rapidly and when 200 micrograms was injected, 96.3 per cent of the dose was excreted. The table also illustrates the remarkable effect the dose size has upon the fetal-maternal relation of the retained radioactivity. With the lowest dose the concentration in the fetal tissues was 130 times higher than the concentration in the maternal tissues.

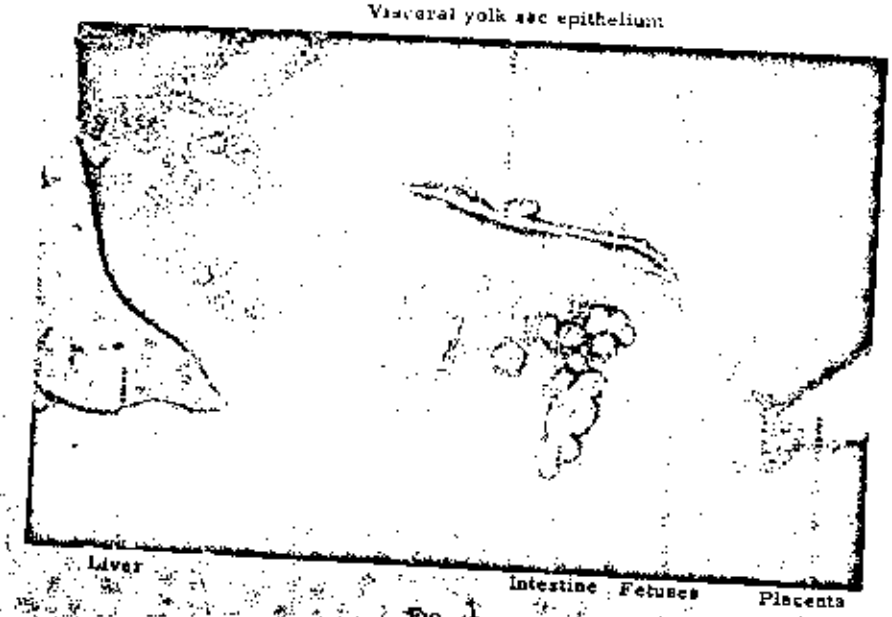

FIG. 4b

Detail of neighbouring autoradiogram to 4a. In the fetus which shows radioactivity the highest concentration is seen in the intestine. In the laterally sectioned placenta an accumulation is seen in the visceral yolk sac epithelium. The radioactive substance has not yet reached the right fetus but its placenta shows high concentration.

The fetal-maternal ratio decreased to 10 with the dose 0.5 micrograms and was less than 2 with the giant dose 0.2 mg.

Tables II and III illustrate the temporal pattern for excretion (the retained activity being measured by whole-body scintillation).

In the experiments in Table II two fairly large doses have been used and the percentage retention followed from 1 hour to 8 hours in pregnant adult mice. With both doses the excretion was high during the first 3–4 hours and then decreased abruptly.

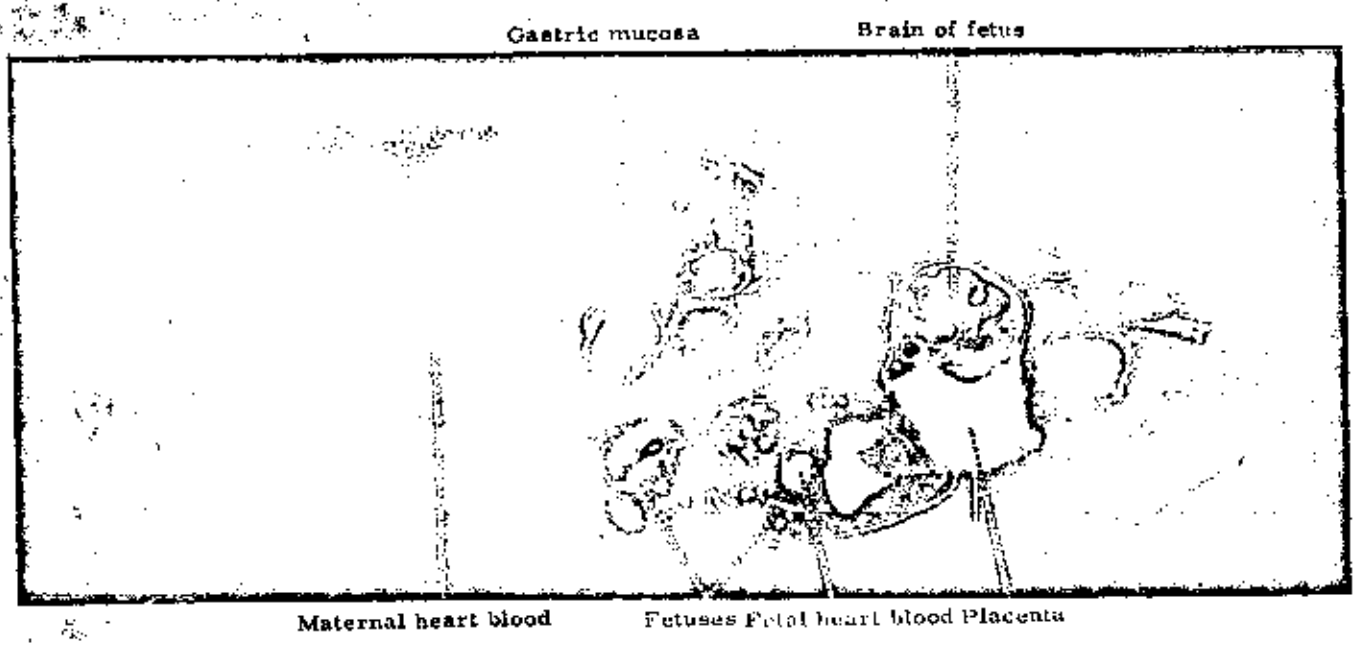

FIG. 5a

Autoradiogram of Co^{58}-vitamin B_{12} in a pregnant mouse 4 hours after intravenous injection. The placentae still have the highest activity. The concentration is now higher in the fetuses than in the mother. The fetal blood level is fairly high while the maternal is very low.

Table III shows the excretion during 6 days in pregnant and non-pregnant mice given smaller doses. The excretion was only slightly greater in the non-pregnant mice.

Fig. 1 and 2 illustrate how the relation between placentae, fetuses and maternal bodies changes with time.

Fig. 1 shows the concentration of radioactive substance per gram tissue of the placentae, the fetuses, and the remaining body tissues of the dam in relation to the mean activity.

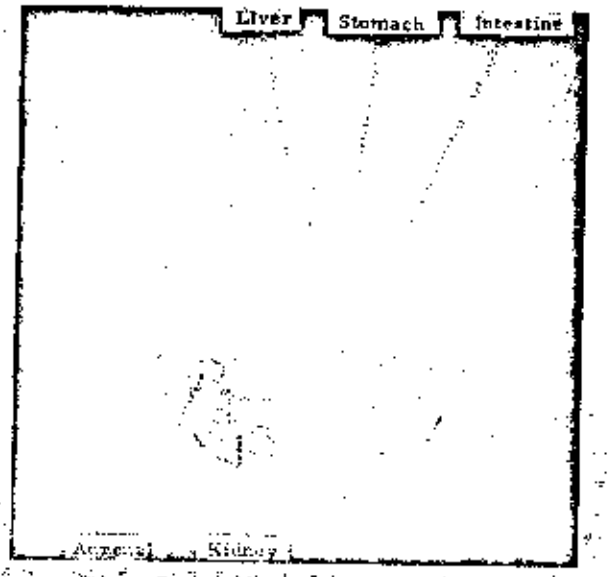

FIG. 5b

Detail of Fig. 5a showing left fetus. Accumulation is seen in kidney, adrenal and gastric mucosa.

The activity in the placentae has within an hour gone up to more than 25 times the mean activity. The concentration in the placentae dominates during the first 8 hours. The fetal concentration passes the maternal by 1½ hours and by 24 hours it has also exceeded the placental level. At this time the activity in the remaining maternal tissues is only about 1/10 of the mean activity mainly due to the accumulation in the fetuses and the placentae. The activity in the fetuses is more than 50 times higher than in the maternal bodies.

Fig. 2 illustrates the changes with time of the whole content of radioactive substance in 1) the collected placentae, 2) the collected fetuses and 3) the remaining maternal tissues. The values are given as per

cent of the entire retained dose (placentae + fetuses + body = 100). The table shows that the placentae from 15 minutes to 2 hours after injection contain more than half of the activity in the whole pregnant mouse. At all times studied the collected placentae contain more radioactive substance than the maternal carcass. At 24 hours the fetuses contain more than 3/4 and the maternal body less than 1/10 of the retained activity.

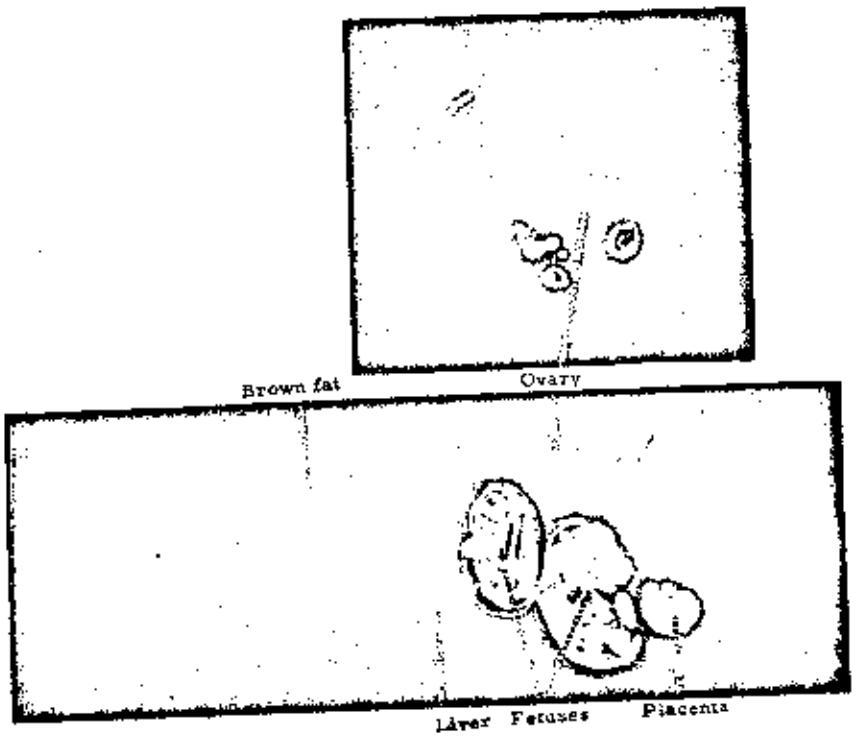

FIG. 6

Autoradiogram of a pregnant mouse 4 days after injection of Co^{58}-labelled vitamin B_{12}. The concentration is very high in the fetuses and placentae but low in the mother. In the mother the highest concentration is found in the follicular walls and corpora lutea of the ovary (see enlarged detail).

Autoradiography.

Uptake in maternal organs. After 15 minutes the uptake in the placentae dominated the distribution picture (FIG. 3). Among the maternal organs an accumulation was also noticed in the cortex of the kidney, in some endocrine organs such as the pituitary, adrenal cortex, thyroid and gonads and in the pancreas, the gastric mucosa and the

liver. The passage to the brain was hindered but high activity was noticed in the choroid plexus and in a small area in the brain stem. Accumulation in these organs was also evident at the later times studied, even in animals killed 16 and 32 days after injection. A rather low uptake was seen in the bone marrow. The main change with time in the distribution pattern was, that at the late times studied relatively high activity was noticed in the brain, and that the brown fat which early showed a modest accumulation later became one of the more dominating tissues in the autoradiograms (FIG. 8).

The distribution between mother, placentae and fetuses during the first day after injection is presented in the scintillation counting tables.

Four days after injection the fetuses still dominated as they did after 24 hours (FIG. 6). The activity of the placentae had decreased but was still high. Even in early pregnancy (10 days after mating and approximately 4 days after implantation) both the placentae and fetuses showed a higher concentration than any maternal tissues (FIG. 7).

After 16 days the concentration level of the placentae and the fetal tissues was similar to that of the maternal tissues (FIG. 8). After 32 days (the animals being injected 12 days before mating) the activity in the fetuses was lower than in the dams.

As far as the distribution in the placentae and fetal membranes is concerned, radioactivity was limited to the chorioallantoic placenta after 15 minutes. Later on, an even stronger accumulation was noticed in a thin layer covering the inner part of the chorioallantoic placenta which probably corresponds to the visceral yolk sac epithelium (FIG. 4*b*).

No activity was observed in the fetal fluids.

Distribution within the fetuses. At the time when activity first appears in the fetuses a high concentration was seen in the lumen of the fetal intestine (FIG. 4). This activity disappeared later. Initially there was also a relatively high concentration in the fetal blood (FIG. 5*a*) (while the maternal blood concentration had gone down to a scarcely perceptible level).

Otherwise, accumulation could be noticed in the same tissues of the fetuses as in the mother. A specific uptake was seen in the cortex of the kidney but no activity was observed in the renal hilus or in the bladder of the fetuses. Specific accumulation was also noticed in the gastric mucosa — especially in the bottom of the crypts (the zymogenic cell layer) (FIG. 5*b*).

From 4 hours to 4 days after injection every fetal tissue including the endocrine organs, pituitary, adrenal cortex, and thyroid, showed

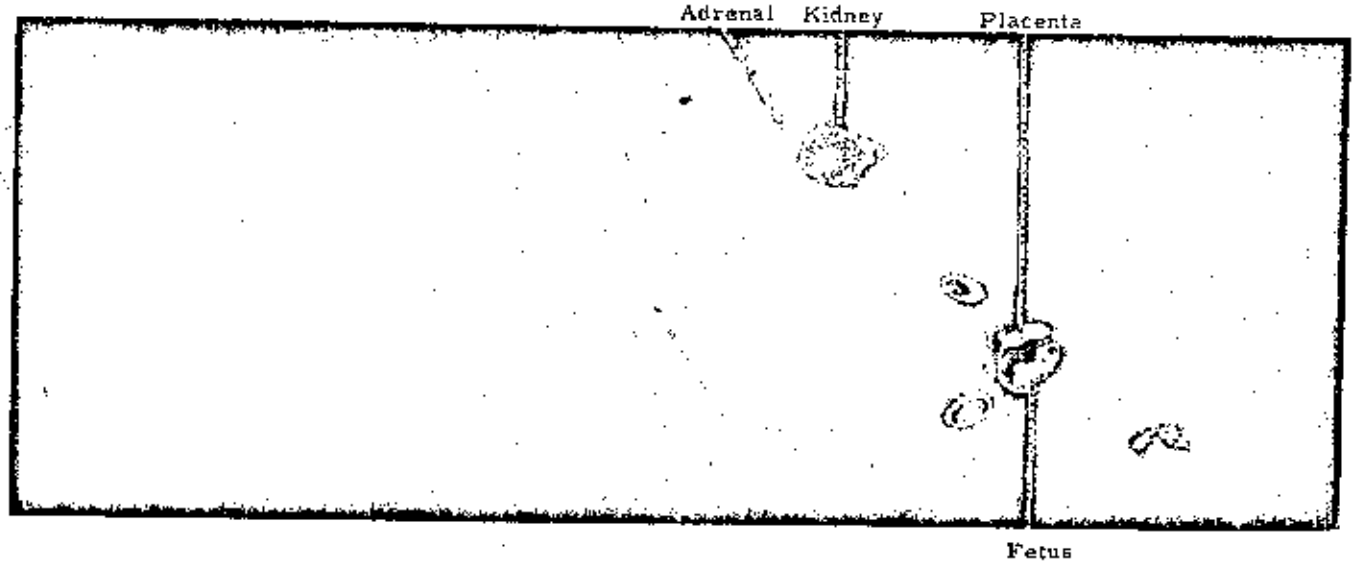

FIG. 7

Autoradiogram showing accumulation of Co^{58}-vitamin B_{12} in placenta and fetus in early pregnancy. Length of fetus 3.5 mm. The mouse is killed 24 hours after injection in a tail vein.

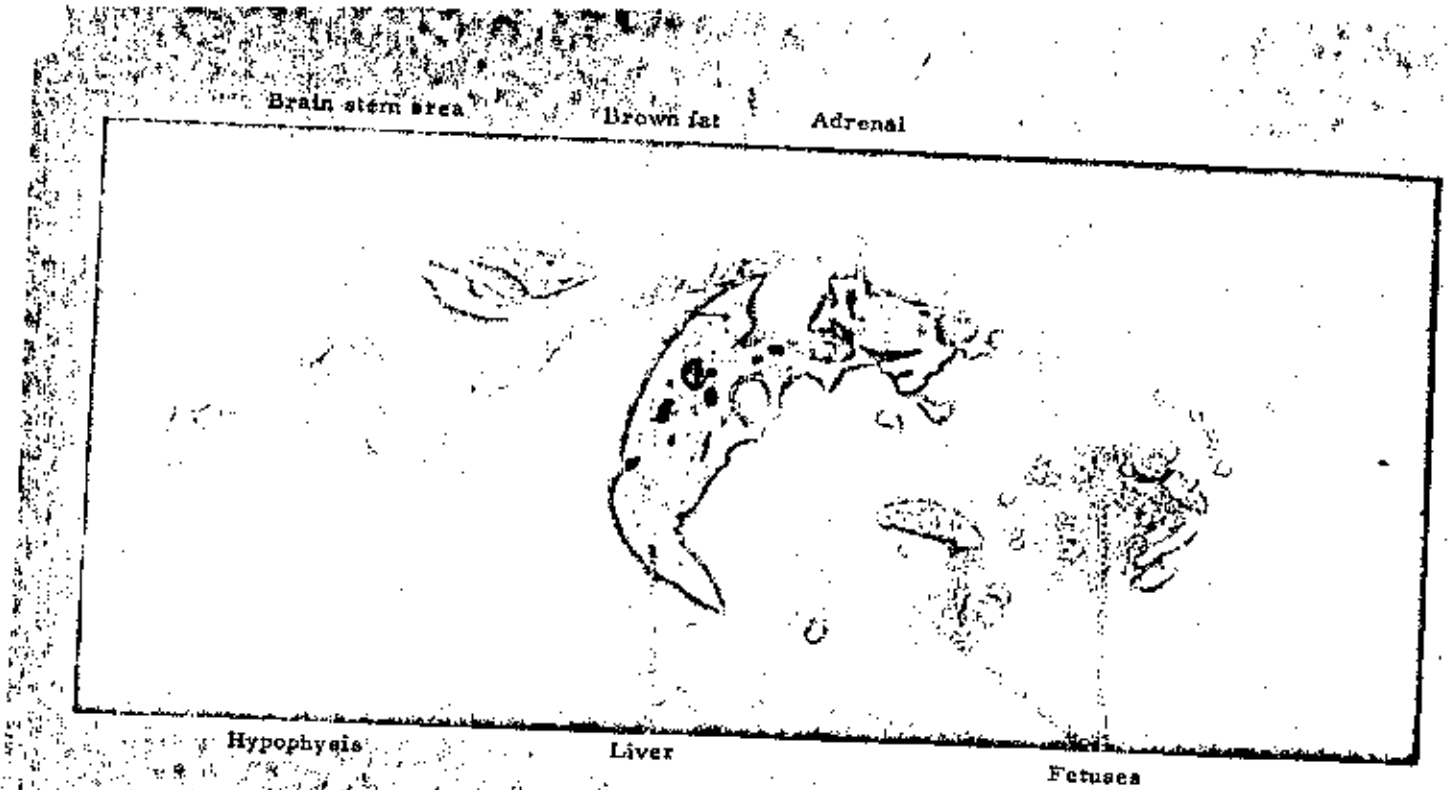

FIG. 8

Autoradiogram showing the distribution of radioactivity in a pregnant mouse 16 days after injection of Co^{58}-vitamin B_{12}. The fetal and placental activity is at this time hardly higher than the average maternal. In the mother accumulation is seen in the "regular" vitamin B_{12} localisation areas e.g. adrenal cortex, hypophysis and the brain stem area but also in brown fat.

a significantly higher concentration than the corresponding maternal tissues. A significant though modest accumulation could be noticed in the liver. The radioactivity levels of the skeletal muscles and the bone marrow were, as in the adult animal, comparatively low. The distribution pattern in the fetuses showed similar changes with time as that of the mother -- with a relative increase in the CNS and brown fat.

The initial accumulation in a brain stem area and in the choroid plexus was also stronger in the fetuses than in the mothers (FIG. 5a). The uptake in the rest of the brain appeared to proceed more rapidly. Mice, given vitamin B_{12} before mating, showed a specific accumulation in the same fetal organs as those mice, given B_{12} during pregnancy, although the over all level was much lower.

DISCUSSION

The vitamin B_{12} molecule is considered to be very stable in the body. A single injected dose has been shown to give a therapeutic effect in man lasting for several months (31, 25). Chromatographic investigations (NEUJAHR and ULLBERG, to be published) have shown that in mice 14 days after intravenous injection the major part of the radioactive substance of the liver still represents intact vitamin B_{12}.

The pattern of transfer from mother to fetus of vitamin B_{12} which has been observed in the autoradiograms is peculiar in several respects, the strong and rapid accumulation in the placenta, the slow further transfer to the fetus, and the final very strong accumulation in the fetuses compared with the mother.

Among the many substances which have been studied in our laboratory using whole-body autoradiography none has behaved similarly. The other vitamins studied (Vitamin A, 30; Vitamin B_1, 12; Vitamin C, 13; Vitamin E, 30) have not been found to accumulate in fetuses. Many other substances of nutritional significance such as amino acids (14), glucose (ULLBERG and KORANSKY, to be published), iron (28) and iodine (29) do accumulate to a slight extent. The placental transfer pattern, however, is different. These substances do not accumulate in the placenta as vitamin B_{12} does and they appear in the fetuses as early as 1–5 minutes after intravenous injection of the mother.

The transfer of vitamin B_{12} through the placenta shows some similarity to the absorption of vitamin B_{12} in the gut of rats according to investigations by BOOTH *et al.* (6) and LATNER *et al.* (17). They found

that perorally administered vitamin B_{12} was rapidly taken up and concentrated in the mucosa of the small intestine. There was, however, a slow further transfer from the intestinal wall to the blood.

The similarities in time sequence between the placental passage and intestinal absorption of vitamin B_{12} may indicate a similar transportation mechanism in the placenta as in the intestine. Intrinsic factor or a similar substance may also play a role in the placental transportation of vitamin B_{12}.

The rapid relative decrease of fetal accumulation with increased B_{12}-dose indicates a limit to the capacity of the presumable active transportation mechanism — similar to what is found for the intestinal absorption of vitamin B_{12}.

A possible reason for a need for an active mechanism of transportation of vitamin B_{12} through the epithelial barriers of both the placenta and the intestine may be the large size of the vitamin B_{12} molecule (molecular weight about 1400).

In earlier reports high concentration of vitamin B_{12} in fetuses has been found by CHOW *et al.* (8) for rats and LUHBY *et al.* (20) and WOODS *et al.* (31) for dogs. A placental concentration mechanism is likely to be present in humans as well. Several investigators have found higher concentration of vitamin B_{12} in fetal cord blood than in the maternal venous blood in pregnant women and a significantly lowered vitamin B_{12}-serum concentration in pregnant women (16, 4, 5, 9, 22, 1, 21, 18).

During pregnancy the maternal share of absorbed vitamin B_{12} will probably be decreased due to the high fetal demand. This may at least partly explain the increased frequency of symptoms of vitamin B_{12}-deficiency which has been noticed during pregnancy (15).

There is apparently a need for clinical investigation to study whether there exists a state of deficient placental transportation (similar to that of the gastrointestinal absorption) of vitamin B_{12}, which can be responsible for cases of fetal death or damage.

Before giving radioactively labelled vitamin B_{12} to pregnant women the probably high radiation dose of the fetus and placenta should be considered.

The autoradiograms show that if vitamin B_{12} is administered to female mice before mating only a small portion of the dose is transferred to the fetuses. Thus, the activity which has already been taken up by the maternal tissues does not to any large extent pass over to the fetuses, indicating that the tissue supply of the mothers is not depleted. This observation agrees with that of LUHBY *et al.* (20), who

found low concentrations in the fetuses after having given vitamin B_{12} to bitches 5 months before mating. The fetuses thus seem to depend on the vitamin B_{12} which is taken up by the mother during pregnancy.

Summary

Radio-cobalt-labelled vitamin B_{12} has been given intravenously to pregnant mice and the distribution has been studied in mother, placenta and fetuses under varying experimental conditions by whole-body autoradiography and scintillation counting.

Injected Co^{58}-B_{12} accumulated strongly and rapidly in the placenta followed by a slow transfer to the fetuses. The peak concentration in the fetuses was not reached until after about 24 hours. The degree of fetal accumulation was dose-dependent. With the smallest dose used (0.02 μg), the average fetal concentration was 130 times higher than the maternal. This unusual pattern of placental transfer indicates a specific transportation mechanism for vitamin B_{12}, possibly similar to conditions in the gastrointestinal tract (involving intrinsic factor or a similar substance).

After having reached the fetuses the radioactive compound was not located in a storage organ but was distributed to all fetal tissues. Both in the mother and the fetuses the highest concentration was found in the endocrine organs, the renal cortex and the gastric mucosa.

Acknowledgments

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The Metabolic Significance of Reduced Serum B_{12} in Folate Deficiency

By Martin B. Van Der Weyden, Mary Rother, and Barry G. Firkin

The depressed serum B_{12} levels accompanying folate deficiency generally increase following folic acid therapy, and this phenomenon is cited as evidence that true B_{12} deficiency did not preexist in such instances. In this study, the metabolic significance of reduced serum B_{12} levels complicating folate deficiency was determined in bone marrow cultures by evaluation of abnormal incorporation of deoxyuridine into DNA-thymine, a defect characteristic of megaloblastic maturation due to folate or B_{12} deficiency. In in vitro marrow cultures of seven patients with folate deficiency and normal serum B_{12} levels, added B_{12} resulted in no change in the depressed incorporation of deoxyuridine into DNA-thymine, with complete correction of the defect by added folate. However, in marrow cultures of five patients with folate deficiency and depressed serum B_{12} levels, added B_{12} produced a partial correction of the defective deoxyuridine incorporation, with complete correction by added folate. In vivo pharmacologic doses of B_{12} in a patient with folate deficiency, but normal serum B_{12} levels, resulted in no alteration in the degree of morphologic megaloblastic maturation or the abnormal deoxyuridine incorporation into DNA-thymine. In contrast, in a patient with both B_{12} and folate deficiency, B_{12} therapy resulted in partial correction of the abnormal deoxyuridine incorporation into DNA-thymine, with simultaneous reduction in the degree of morphologic megaloblastic maturation. However, abnormal deoxyuridine incorporation into thymine-DNA, consequent to folate deficiency, persisted. Similar findings were obtained in a patient with folate deficiency and associated reduced serum B_{12}. It is suggested that the depressed serum B_{12} in patients with folate deficiency represents a true deficiency for hemopoietic tissue and contributes to the megaloblastic maturation by its effect on folate metabolism.

CO-EXISTENT, REDUCED SERUM B_{12} levels below the normal range occur in approximately 40–60% of patients with megaloblastic anemia consequent to folate deficiency.[1-3] Although truly independent B_{12} levels cannot be entirely excluded, the spontaneous increase in serum B_{12} levels following folate therapy[1,7] is cited as evidence that true B_{12} deficiency does not exist under these circumstances.[4] Suggested mechanisms responsible for the production of depressed serum B_{12} levels include malabsorption of B_{12}[5,6] or

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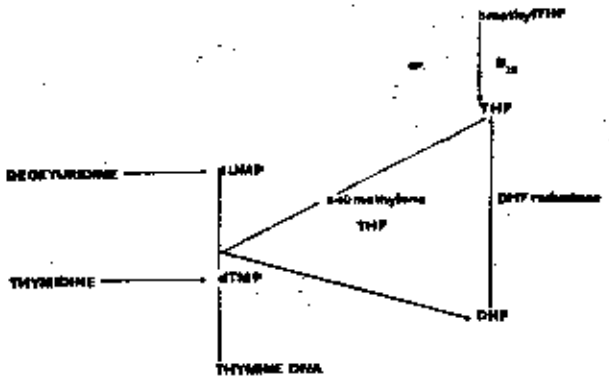

Fig. 1. Synthesis of DNA-thymine from deoxyuridine monophosphate (dUMP). Points of involvement of vitamin B_{12} and folate intermediates are indicated. dTMP, thymidine monophosphate; THF, tetrahydrofolic acid; DHF, dihydrofolic acid.

redistribution of B_{12} within the body.[7,8] Zalusky et al.[24] reported that, in patients with uncomplicated folate deficiency, pharmacologic doses of B_{12} were capable of inducing a reticulocyte response, despite little change in the degree of morphologic megaloblastic maturation. The metabolic consequences, however, of reduced serum B_{12} levels in folate-deficient states at the cellular level, or the contribution of depressed levels to megaloblastic maturation, remain speculative.

A sensitive indicator of folate or B_{12} deficiency at the cellular level is the interference with effective DNA-thymine synthesis from deoxyuridine (dU) in short-term in vitro human bone marrow cultures.[9–11] In normoblastic short-term cultures, added dU enters the deoxyuridine monophosphate (dUMP) → thymidine monophosphate (dTMP) → DNA-thymine pathway and suppresses the incorporation of subsequently added tritiated thymidine (^{3}H-TDR) into DNA.[10,11] The interrelated biochemical pathways are shown in Fig. 1. Interference with the dU suppressive effect is abnormal and is a reflection of reduced tissue folate levels.[10]

This paper reports studies of abnormalities of synthesis of dTMP from dU in in vitro bone marrow cultures from patients with folate deficiency exhibiting associated normal or reduced serum B_{12} levels, and the effect of pharmacologic doses of B_{12} in vivo in modifying the abnormal dU suppressive effect in folate deficiency associated with normal serum B_{12} levels or those below the normal range.

MATERIALS AND METHODS

Effective synthesis of dTMP from dU in human marrow was measured by the ability of preincubation with unlabeled dU to suppress incorporation into DNA of subsequently added ^{3}H-TDR, as described previously.[10,11] In this system, abnormal synthesis in megaloblastic anemia is demonstrable by reduced ability of preincubation with dU for 1 hr at room temperature to suppress incorporation of subsequently added ^{3}H-TDR. For bone marrow culture, 15–20 ml of marrow were aspirated directly into 10 ml of cold Hank's solution containing heparin, 100 U/ml. All operations were carried out as previously described.[11] The radioactive precursor used was thymidine (methyl-^{3}H, specific activity 26 Ci/mM, Radiochemical Center, Amersham, England) prepared as a solution containing 10 μCi/ml. The dU load was 10^{-3} μmole/ml, concentration of added B_{12} (cyanocobalamin) 1 μg/ml and folic acid (PGA) 50 μg/ml. Marrow cultures were performed in triplicate,

and dU, folic acid, or B_{12} was added to each set of cultures. ^{3}H-TDR incubation (1 μCi/ml) was performed at 37°C for 2 hr, and DNA was extracted from the precipitate by the technique of Feinendegen et al.[12] as modified by Cooper and Rubin.[13] The radioactivity of the DNA extract was measured in a liquid scintillation counter (Nuclear Chicago Corp., Des Plaines, Ill.), and results were expressed as total radioactivity incorporated into DNA. At the time of aspiration of bone marrow samples, venous blood was assayed for serum B_{12}[14] and folate.[15] The normal range of serum B_{12} is 200–800 pg/ml and that of serum folate is 3.2–20.0 ng/ml.

The patients studied consisted of seven patients (No. 1–7) with megaloblastic maturation associated with reduced serum folate below the normal range but normal serum B_{12} levels, and five patients with folate deficiency and associated reduced serum B_{12} levels below the normal range. The latter group of patients (No. 8–12) exhibited normal response in gastric secretion to pentagastrin[16] or tubeless gastric analysis, and were negative for gastric parietal antibody.[18] The clinical details are shown in Table 1. In the patients with nutritional megaloblastic anemia, the diet was evaluated by the hospital dietitian and one of the authors and was considered inadequate to maintain folate stores, but was not deficient in B_{12}.

RESULTS

In normoblastic marrow cultures, added dU enters the dUMP → dTMP → DNA-thymine pathway, so that the incorporation of subsequently added ^{3}H-TDR into DNA is reduced to 10% or less of the control marrow cultures, i.e., cultures not preincubated with dU.[10,11] The abnormal dU suppression of ^{3}H-TDR incorporation into DNA in marrow cultures obtained from patients with folate deficiency is shown in Fig. 2. In patients with folate deficiency but normal serum B_{12} levels (No. 1–7), Fig. 2A shows that the addition of B_{12} to the marrow cultures resulted in no significant correction of the defective dU suppression, with complete correction (less than 10%) by the addition of folic acid. In contrast, the addition of vitamin B_{12} to the marrow cultures obtained from patients with folate deficiency and reduced serum B_{12} levels below the normal range (No. 8–12) produced a partial correction of the metabolic defect, with complete correction by the addition of folic acid (Fig. 2B). The two groups of patients with or without reduced serum B_{12} levels below the normal range may not be strictly comparable. The patients with reduced B_{12} levels (No. 8–12) included four patients with nutritional anemia and a patient with epilepsy (on Dilantin therapy). In this group of patients, the degree of abnormal dU suppression was particularly uniform (see Table 1). The group of patients with normal serum B_{12} levels (No. 1–7) were less uniform in clinical status, including three patients with alcoholic liver disease. The degree of abnormal dU suppression was similarly less uniform, varying from 18 to 77%, and serum B_{12} levels ranged from near the lower range of normal to above the normal range (Table 1). The uniform, in vitro response to B_{12} in marrow cultures in the two groups of patients makes it unlikely that the difference in experimental observation between the two groups was due to factor(s) other than the difference in B_{12} levels.

The effect of pharmacologic doses of B_{12} in ameliorating megaloblastic maturation due to folate deficiency was studied in one patient (No. 3)—with reduced folate below the normal range but normal serum B_{12}—who, during the study, was admitted to a general ward but maintained on a diet similar to

Table 1. Clinical Details of Patients Studied

Patient Number	Diagnosis	Marrow Morphology	Serum* Vitamin B_{12}	Serum Folate†	dU Suppression of ^{3}H-TDR into DNA‡
					(%)
1	Alcoholic cirrhosis	Megaloblastic	250	1.0	34
2	Alcoholic cirrhosis delirium tremens	Megaloblastic	290	2.0	51
3	Nutritional megaloblastic anemia	Megaloblastic	260	1.4	77
4	Alcoholic cirrhosis	Megaloblastic	1110	1.2	38
5	Nutritional megaloblastic anemia	Megaloblastic	210	1.2	57
6	Lymphosarcoma, Coombs' positive haemolytic anemia	Early megaloblastic	360	1.4	18
7	Chronic renal failure	Early megaloblastic	300	1.2	22
8	Nutritional megaloblastic anemia	Megaloblastic	110	1.0	60
9	Nutritional megaloblastic anemia	Megaloblastic	110	1.8	44
10	Rheumatoid arthritis, nutritional megaloblastic anemia	Megaloblastic	110	1.0	48
11	Nutritional megaloblastic anemia	Megaloblastic	125	2.0	37
12	Epilepsy	Megaloblastic	115	2.4	39

* Range of normal serum B_{12} = 200–600 pg/ml.
† Range of normal folate = 3.2–20 ng/ml.
‡ dU suppression of ^{3}H-TDR in DNA in normoblastic marrow cultures = <10%.

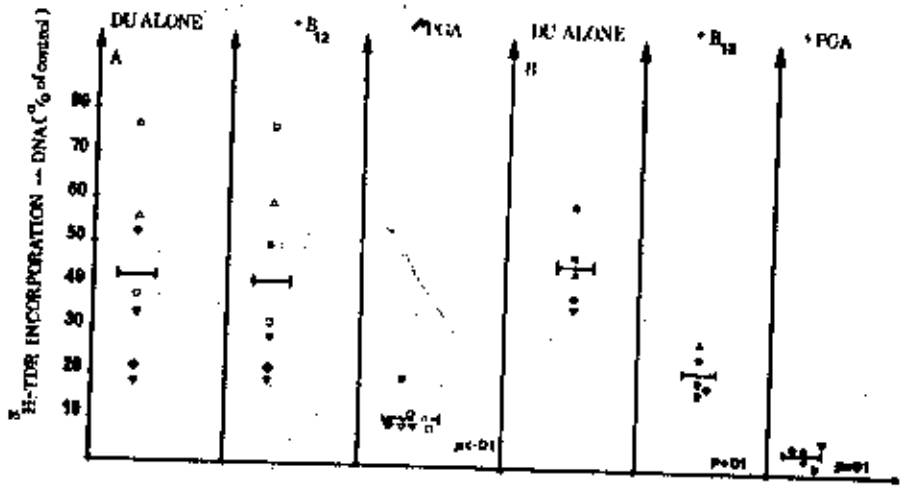

Fig. 2. Effect of folic acid and vitamin B_{12} on dU suppression of ^{3}H-TDR incorporation into DNA in marrow cultures from patients with folate deficiency and normal serum B_{12} levels (Fig. 2A), and with folate deficiency and reduced serum B_{12} levels (Fig. 2B). Deoxyuridine (dU), 10^{-1} μmole; ^{3}H-TDR, H^3-thymidine; folic acid, 50 μg/culture; and B_{12}, 1 μg/culture. Mean indicated by horizontal bars.

prehospitalization. During a control period of 3 days, the reticulocyte count ranged from 0.3 to 0.4%. Serum iron was 200 μg/100 ml total iron binding capacity (T.I.B.C.) 270 μg/100 ml, serum folate 1.4 ng/ml and B_{12} 260 pg/ml. The marrow morphology was frankly megaloblastic, and dU suppression of ^{3}H-TDR into DNA was 77% (normal less than 10%), with no effect by the addition of B_{12} to the marrow cultures and complete correction by the addition of folate. Subsequently, B_{12} (cyanocobalamin) was administered i.m., 500 μg/day during an 8-day period. Repeat marrow biopsy at the completion of B_{12} therapy exhibited no change in the morphologic degree of megaloblastic maturation, and the dU suppression was unchanged at 79%. Reticulocyte count during the B_{12} administration ranged from 0.2 to 0.5%. Other parameters at the time of repeat marrow biopsy were: serum iron, 200 μg/100 ml; T.I.B.C., 210 μg/100 ml; serum folate, 1.0 ng/ml; and serum B_{12}, 3600 pg/ml. After 3 days with no change in the reticulocyte count, folic acid therapy (50 μg i.m./day) resulted in a reticulocytosis reaching 41%.

In contrast, in a patient reported previously[11] with pernicious anemia and associated folate deficiency (serum B_{12}, 20 pg/ml; serum folate, 1.8 ng/ml), the marrow, prior to treatment with B_{12}, exhibited abnormal dU suppression (62%) with partial correction by the addition of B_{12} (48%) and further correction by the addition of folic acid (20%). Ninety-six hours after B_{12} therapy, 500 μg/day, the reticulocyte count had risen from 3% to 15%, and repeat marrow revealed a reduced degree of morphologic megaloblastosis and amelioration of the abnormal dU suppression (29%). Addition of B_{12} to the marrow culture, in contrast to pre-B_{12} therapy, was without effect (dU suppression 33%) with complete correction by the addition of folic acid (5%). Serum B_{12}

at the time of repeat marrow was 2300 pg/ml, and folate 0.9 ng/ml. A similar pattern was demonstrated in a patient (No. 12) with folate deficiency and reduced serum B_{12} below the normal range. The marrow prior to treatment with B_{12} exhibited abnormal dU suppression (39%) with partial correction with the addition of B_{12} (18%) and complete correction with folate (6%). Forty-eight hours after commencing B_{12} therapy (500 μg/day), repeat marrow aspiration revealed a moderate reduction in megaloblastosis and associated reduction in abnormal dU suppression (20%), with no significant correction by the addition of B_{12} (18%) and complete correction by the addition of folate (2%).

DISCUSSION

In 142 patients with megaloblastic anemia due to folate deficiency, reported by Mollin et al.,[1] 47% exhibited depressed serum B_{12} levels and, in 10%, the level was less than 100 pg/ml. Similarly, six of ten patients reported by Hansen and Wienfield[3] and 12 of 21 patients with folate deficiency reported by Cooper and Lowenstein[8] had associated subnormal B_{12} levels. In contrast, erythrocyte B_{12} levels are invariably low in folate deficiency.[19] The effect of folate therapy on the depressed serum B_{12} levels is variable but generally produces a rise in the serum B_{12}. Mollin et al.[1] reported that the serum B_{12} failed to rise consequent to folate therapy only in patients with coexistent malabsorption of B_{12}. In the data reported by Johnson et al.,[7] despite a significant rise in serum B_{12} levels in 12 of 21 patients with tropical malabsorption reaching a peak 3–17 days after commencing folic acid therapy, in only five of the 11 patients was this sustained at 30 days, and in only three was the serum B_{12} in excess of 150 pg/ml. The subnormal erythrocyte B_{12} levels in folate deficiency return to normal following therapy.[19] However, in patients exhibiting associated reduced serum B_{12} levels, the erythrocyte B_{12} level failed to be sustained in the normal range following folic acid therapy.[19] The mechanisms responsible for the depressed erythrocyte or serum B_{12} levels below the normal range or for the subsequent rise following folic acid therapy are speculative and unproven.

The contribution of the depressed serum B_{12} in folate deficiency to the megaloblastic maturation is demonstrated by this study. In marrow cultures obtained from patients with B_{12} deficiency and associated normal or increased serum folate levels, the abnormal incorporation of dU into DNA-thymine is partly corrected by added B_{12} and completely corrected by folic acid.[10,11] In contrast, in pure folate deficiency, added B_{12} is without effect. The pattern exhibited by patients with folate deficiency with coexistent, depressed serum B_{12} levels is similar to that obtained in patients with uncomplicated B_{12} deficiency, namely partial correction of the abnormal dU incorporation into DNA by B_{12} with complete correction by folic acid.

These findings may be explicable on the basis that the reduced serum B_{12} levels in folate deficiency represent for hemopoietic tissue a true deficiency at that point in time. Consequently, the resultant impaired conversion of 5-methyl-tetrahydrofolic acid (5M-THF) to tetrahydrofolic acid (THF), a B_{12}-

dependent step,[10,20,21] and the decreased cellular uptake of 5M-THF, possibly B_{12}-dependent,[22] potentiate the degree of depleted tissue levels of active folate intermediates, with ensuing reduced conversion of dUMP to dTMP, a folate-dependent step.[23] This concept is supported by the finding in the patients with both B_{12} and folate deficiency, where the administration of B_{12} resulted in the conversion of the metabolic defect consequent to B_{12} deficiency, with coexistent reduction in megaloblastosis. However, evidence of abnormal dU incorporation into DNA, consequent to folate deficiency, persisted. In contrast, pharmacologic doses of B_{12} in a patient with folate deficiency but normal serum B_{12} were without effect in reducing the degree of megaloblastosis or abnormal dU incorporation into DNA and were ineffective in inducing a reticulocytosis. This variance from the findings of Zalusky et al.[24] may be explicable on the basis that the patients studied by these workers had alcoholic liver disease and may have had tissue depletion of B_{12} despite normal serum B_{12}, a phenomenon demonstrable in alcoholic liver disease.[25]

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VITAMIN B_{12} AND NUCLEIC ACID BIOSYNTHESIS*

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While there is much evidence that folic acid is concerned with the use of 1-carbon metabolic intermediates in purine biosynthesis (1), a role of vitamin B_{12} in this pathway has not been established despite its role in the 1-carbon metabolism of methyl synthesis (2). Postulations as to such a role for vitamin B_{12} in nucleic acid synthesis are based practically entirely on the fact that some vitamin B_{12}-requiring organisms will grow when given thymidine and other nucleotides in the absence of known added vitamin B_{12} (3, 4). The present investigation deals with the study of incorporation of 1-carbon precursors and C^{14}-glucose into nucleic acids of liver of both vitamin B_{12}-deficient and normal pigs, chicks, and rats (5).

EXPERIMENTAL

Production of Vitamin B_{12} Deficiency in Animals—Vitamin B_{12} deficiency was produced both in baby pigs and chicks as reported previously from this laboratory (6, 7). In the case of rats, the deficiency was produced by feeding the soy bean-lactose diet of Cuthbertson and Thornton (8). Normal baby pigs and rats received 1 γ of vitamin B_{12} per kilo of body weight per day by injection, while chicks received 50 γ of vitamin B_{12} per kilo of diet. The vitamin B_{12}-deficient and normal animals were injected with 40 μc. per kilo of body weight of the following: formate-C^{14} (specific activity 8.9 mc. per mmole), formaldehyde-C^{14} (specific activity 4 mc. per mmole), glycine-2-C^{14} (specific activity 1.0 mc. per mmole), serine-3-C^{14} (specific activity 1.8 mc. per mmole), $C^{14}H_3$-methionine (specific activity 0.42 mc. per mmole), and uniformly labeled glucose (specific activity 1.5 mc. per mmole). The animals were killed after 4 hours, and the livers were removed and analyzed for RNA and DNA.[1] In the experiments for urinary allantoin isolation, the rats were injected with the radioactive precursors shown in Table II, and 10 day urine collections were made for each rat.

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[1] The following abbreviations are used: RNA, ribonucleic acid; and DNA, deoxyribonucleic acid.

Isolation of Nucleic Acids and Allantoin—Nucleic acids were isolated and separated by the method of Bendich *et al.* (9). They were then freed of glycogen by subjecting it to amylase treatment and of nucleoprotein by adjusting the material to pH 4.2. The DNA and RNA were then dissolved in water and plated to infinite thinness, and the radioactivity of samples was determined in a gas flow counter. The results are given in Table I. Allantoin was isolated by the method of Brown *et al.* (10). This crystalline allantoin was plated at infinite thinness, and the radioactivity was determined as above. The results are given in Table II.

Results

The data in Table I show that there was no difference in the radioactivity of RNA and DNA from vitamin B_{12}-deficient and normal animals. The radioactivity of the nucleic acids from the formate- and formaldehyde-injected animals was higher than that from the animals given other precursors (such as glycine, serine, and methionine); however, there was no difference between vitamin B_{12}-deficient and normal animals. Similarly, when serine-3-C^{14} was injected into the normal and deficient rats, no difference was observed in the radioactivity of the nucleic acids (plus vitamin B_{12}, 230 c.p.m. per mg.; minus vitamin B_{12}, 242 c.p.m. per mg.). The results were similar for chicks, pigs, and rats. The observations made with uniformly labeled glucose-C^{14} also showed no differences in the activity of the nucleic acids between normal and deficient pigs. Similarly, there was no difference in the labeling of the allantoin excreted by vitamin B_{12}-deficient and normal rats.

DISCUSSION

The results clearly indicate that there is no difference in the amount of incorporation of 1-carbon metabolites into nucleic acids in vitamin B_{12}-deficient and normal animals. Rose and Schweigert (11) isolated both RNA and DNA from the livers and other tissues of rats fed vitamin B_{12}-deficient and normal rations, and they found that the total DNA content per gm. of liver was reduced by approximately 20 per cent in the vitamin B_{12}-deficient animals as compared to control animals, while RNA was reduced by approximately 10 per cent. This may possibly be due to the differences in food intake and final body weight.

The total radioactivity incorporated into nucleic acid compares well with that reported by Haydar *et al.* (12) using C^{14}-formate or glycine and that of Barclay *et al.* (13). In the present studies the incorporation of the C^{14} compounds into RNA and DNA was very nearly the same, which was somewhat surprising in that DNA has a slower turnover rate than RNA (14). This is somewhat clarified in the data from the time studies of

Haydar and coworkers (12). According to them, maximal incorporation in RNA in the intact animal is reached at the end of the first 2 hours and then falls off rapidly. In the present studies, the animals were sacrificed after 4 hours; thus, the most probable explanation is that the incorporation of radioactivity from a 1-carbon precursor was still increasing in the case of DNA even at the end of 4 hours, as shown by Friedkin et *al.* (15), for thymidine-C^{14} incorporation, while that in RNA was decreasing owing to catabolism after the peak had been reached within the first 2 hours, the net result being similar labeling of RNA and DNA. Similarly, Barclay

TABLE I

Effect of Vitamin B_{12} on Nucleic Acid Synthesis

Precursor administered	Activity in isolated nucleic acids, c.p.m. per mg.			
	Complete diet		Vitamin B_{12}-free diet	
	RNA	DNA	RNA	DNA
Chicks				
Formate-C^{14}	680	605	625	582
Formaldehyde-C^{14}	590	482	470	505
Glycine-2-C^{14}	460	328	388	305
Serine-3-C^{14}	308	325	330	330
Methionine-$C^{14}H_3$	440	360	409	320
Pigs				
Formate-C^{14}	360	316	318	356
Serine-3-C^{14}	266	282	266	226
Glucose-C^{14}	255	260	230	235

et al. (13) observed no difference in activity in the isolated adenine, guanine, and thymine from DNA and adenine and guanine from RNA.

Rege and Sreenivasan (16) and Downing and Schweigert (4), working with vitamin B_{12}-requiring organisms, have shown that both folic acid and vitamin B_{12} are concerned in some way with the control of nucleic acid synthesis. On the other hand, Bergmann *et al.* (17) demonstrated that vitamin B_{12} has no effect on the utilization of the formyl compound (4-formamidoimidazole-5-carboxamide) for purine biosynthesis by *Escherichia coli*. Downing and Schweigert (4), using *Lactobacillus leichmanii*, have shown that vitamin B_{12} is involved in the biosynthesis of deoxyribose. On the other hand, our studies with C^{14}-glucose indicate that vitamin B_{12} is not directly involved either in the conversion of glucose

to ribose or deoxyribose or in the incorporation of these pentoses into nucleic acid in animals. These data may possibly all be explainable by the role of vitamin B_{12} in protein (hence in enzyme) biosynthesis (18, 19).

The results in Table II show that vitamin B_{12} has no direct role in the biosynthesis of the purine ring in the rat. The present work would appear also to rule out any role of vitamin B_{12} in the formation of the functional form of folic acid that is required for incorporation of a 1-carbon unit into purines.

The results obtained when C^{14}-methylmethionine was injected afford additional evidence against the role of vitamin B_{12} in the transmethylation (20). If vitamin B_{12} were involved in transmethylation, it would seem reasonable to suppose that the oxidation of methyl groups would be enhanced in the absence of vitamin B_{12}, giving rise to an increased specific activity in allantoin.

TABLE II

Ability of Rats on Vitamin B_{12}-Free and Complete Diets to Incorporate Various Precursors into Allantoin (Rats)*

Precursor administered	Dose, c.p.m. per day (for 7 days)	Specific activity of allantoin, c.p.m. per mg	
		Complete diet	Vitamin B_{12}-free diet
	× 10^6		
Glycine-2-C^{14}	1.45	773	780
Methionine-$C^{14}H_3$	1.65	472	576
Formate-C^{14}	2.0	4777	5473
Serine-3-C^{14}	1.87	2313	2130

* Three rats in each group.

SUMMARY

The role of vitamin B_{12} in nucleic acid biosynthesis has been studied by the use of various labeled nucleic acid precursors. The results obtained show that vitamin B_{12} is not directly involved in the synthesis of purines and pyrimidines from 1-carbon precursors such as formaldehyde, formate, glycine, serine, and methionine. Studies with C^{14}-glucose also indicate that it is involved neither in the conversion of glucose to ribose or deoxyribose nor in the incorporation of these pentoses into nucleic acids.

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Vitamin B_{12} and Protein Biosynthesis*†

VI. RELATION OF VITAMIN B_{12} TO AMINO ACID ACTIVATION

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It has been reported (1–5) from this laboratory that vitamin B_{12} functions *in vivo* and *in vitro* in the incorporation of amino acids into protein. There was observed a reduction in the incorporation of amino acids into protein in vitamin B_{12}-deficient pigs and rats *in vivo* as compared to normal animals (4). Similar reduction in the incorporation of amino acids was observed upon incubation of liver and spleen homogenates (obtained from vitamin B_{12}-deficient rats after the removal of the nuclei and mitochondria) with adenosine triphosphate, guanosine triphosphate, and fructose diphosphate (ATP,[1] GTP, and FDP) and C^{14}-amino acids (2, 4). Confirmatory data have recently been obtained with chicks (6).[2] A study of the distribution of Co^{60}-labeled vitamin B_{12} administered to a rat showed it to occur principally in the microsomes and 105,000 × *g* supernatant fraction (2, 7). In this paper we present data on the ammonium sulfate fractionation of "pH 5 enzymes" and on the role of B_{12}-containing ones (20 to 60 per cent ASF) in amino acid incorporation into protein.

The work of Keller and Zamecnik (8) has revealed that an enzyme system from rat liver catalyses the incorporation of labeled amino acids into protein under anaerobic conditions. This system consisted of the liver fractions, the soluble cell fraction and the microsomes to which are added C^{14}-amino acid, ATP and an ATP regenerating system such as phosphoenolpyruvate and pyruvate kinase. This simplified system is used in many of the studies reported here.

EXPERIMENTAL

Production of Deficiency: Rats—Weanling male rats of the Sprague-Dawley strain weighing 30 to 40 gm. were fed *ad libitum* the soya flour lactose basal ration previously described (4). The animals were housed individually in an air-conditioned room and maintained for a period of 10 to 12 weeks. The rats on the deficient diet grew slowly and developed anemia as reported previously (4).

Preparation of Microsomes and pH 5 Enzymes—Approximately 15 gm. of rat liver were homogenized, 5.0 gm. at a time, with 2.5 times their volume of buffered medium as described previously (4). The homogenate was centrifuged at 8000 × *g* for 10 minutes at 0°. The supernatant fluid containing the microsomal and soluble fractions was removed and centrifuged at 105,000 × *g* for 1 hour. The supernatant fluid containing the soluble cell fractions was decanted for the preparation of the pH 5 enzymes. The microsomal pellets were homogenized with the same volume of buffered medium and the resulting homogenate was centrifuged again at 105,000 × *g*. The microsomes obtained after the centrifugation were homogenized with buffered medium to give a microsomal suspension containing 25 mg. of protein per ml. The second washing of the microsomes was required to reduce the concentration of the pH 5 enzymes in the microsomes.

To prepare pH 5 enzymes, the supernatant fluid from the first centrifugation at 105,000 × *g* was diluted with an equal volume of unbuffered medium (containing 0.9 M sucrose, 0.004 M $MgCl_2$, and 0.025 M KCl) and the *p*H was carefully adjusted to 5.2 by adding *N* acetic acid drop by drop with constant stirring. The solution was kept at 0° during this procedure. The precipitate was collected by centrifugation in the cold and washed once by suspension in unbuffered medium followed by centrifugation. The precipitate was then dissolved in buffered medium (pH 7.4) to give a solution of the pH 5 enzymes containing 10 mg. of protein per ml. The microsomes and pH 5 enzymes prepared in this way were used in all the studies reported here.

Determination of Protein—Protein concentrations were determined turbidimetrically with TCA. 0.25 to 2.5 mg. of protein in 5.0 ml. of 5 per cent TCA were mixed thoroughly and read at 540 mμ in a Beckman spectrophotometer 30 seconds later. A standard calibration was carried out with known quantities of bovine serum albumin (crystalline) obtained from Armour and Co. This method was used in all the protein determinations.

Incorporation Experiments with pH 5 Enzymes: Microsome System—For the incorporation of 2-C^{14}-alanine into protein the incubations were carried out as described in Table I. The mixtures were incubated (under 5 per cent CO_2 in N_2) for 1 hour at 35°. After the incubation the proteins were precipitated with 10 per cent TCA and were washed and assayed for C^{14} activity as described previously (4).

Distribution of Co^{60}-B_{12} in Liver Fractions—As the previous studies (2) had indicated that over 60 per cent of the vitamin B_{12} was present in the microsome plus supernatant fractions, the

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[1] The abbreviations used are: TCA, trichloroacetic acid; ATP, adenosine triphosphate; GTP, guanosine triphosphate; FDP, fructose diphosphate; ASF, ammonium sulfate fraction; B_{12} antagonist, sulfide of the monocarboxylic acid of vitamin B_{12}.

TABLE I

Activity of B_{12}-containing "pH 5 enzymes" enzyme system

Enzyme system	B_{12} status of rats*	
	$+B_{12}$	$-B_{12}$
	c.p.m./mg. of protein	c.p.m./mg. of protein
1. Complete system†	88	28
2. Complete system + crystalline B_{12}	92	68
3. Complete system + 2 mg. ribonucleic acid	94	31
4. Complete system minus pH 5 enzymes	26	22
5. Complete system minus pH 5 enzymes + crystalline B_{12}	38	30
6. Complete system minus microsomes	20	18
7. Complete system minus microsomes + crystalline B_{12}	21	20
8. Microsomes + pH 5 enzymes from deficient added to normal and normal added to deficient	51	78

* Two rats were used for each study and run in duplicate.

† Complete system contained 0.3 ml. of microsomes; 0.2 ml. of pH 5 enzymes; 0.25 μmole of 2-C^{14}-alanine; 10 μmoles of phosphoenol pyruvate; 0.02 mg. of pyruvate kinase; 0.5 μmole ATP; and 0.25 μmole GTP. The incubation mixture was made 1.0 ml. by the addition of 0.15 M KCl. The incubation mixture 8 contained all other components as in the complete system.

subcellular B_{12} distribution has been studied further. For this purpose, 3 μc. of Co^{60}-vitamin B_{12} was injected into a rat in three doses of 1 μc. each at 8-hour intervals. The rat was killed 8 hours after the last injection. The liver was removed and immediately chilled at 0°, and homogenized with 2.5 times its volume in sucrose buffer. The microsomes and pH 5 enzymes were prepared as described previously except that the microsome preparations were not washed for the second time before taking a sample for counting. As a large amount of activity was found to be present in these microsomes, they were further washed twice. These washings were found to remove considerably more radioactivity, as can be seen in Table II.

Incorporation of Co^{60}-B_{12} in pH 5 Enzymes in Vitro—As vitamin B_{12} was found to be present in pH 5 enzymes (Table II) and since vitamin B_{12} added to the deficient microsomal supernatant preparations gave marked stimulation of amino acid incorporation into protein, it appeared that such an enzyme system might be able to incorporate vitamin B_{12} into its functional site. To study this, three incubations with radioactive B_{12} were carried out: (1) microsomes plus supernatant, (2) microsomes alone, and (3) supernatant alone. In all the cases, ATP, GTP, FDP, and the Co^{60}-B_{12} were added to the incubation mixture. After incubation for 1 hour the mixtures were chilled to 0° and separation of microsomes and pH 5 enzymes carried out as described previously. The fractions obtained were treated with 10 per cent TCA and were washed with hot alcohol and ether, and then counted to measure Co^{60}-B_{12} incorporation. The results are given in Table III.

TABLE II

Distribution of Co^{60}-B_{12} in liver fractions

Fractions	Total c.p.m.	c.p.m./mg. of protein
Whole homogenate	92,080	
Nuclei	12,350	
Mitochondria	15,720	
Microsomes	35,520	613
Microsomes (washed twice)	23,160	400
Supernatant	19,200	
pH 5.2 enzymes	14,260	1,174
Supernatant after pH 5.2 enzyme separation	3,020	
Total nucleic acids	None	

Ammonium Sulfate Fractionation of pH 5 Enzymes—250 gm. of normal rat liver obtained fresh from rats killed by decapitation were chilled in ice and homogenized in 750 ml. of ice-cold buffer medium using a Waring Blendor. All steps in these studies were carried out at 0°. Homogenization was done at full speed for 1 minute. The homogenate was centrifuged for 15 minutes at 15,000 × *g* in a refrigerated centrifuge (Servall SS-1 model). The supernatant obtained was passed through several layers of cheesecloth to remove fat. The supernatant thus obtained was then centrifuged at 105,000 × *g* for 1 hour. The microsomes were discarded and the supernatant was again passed through cheesecloth for removal of residual fat to give Supernatant I. Supernatant I was then adjusted to pH 5.2 as described previously. The precipitate thus obtained after centrifugation at 10,000 × *g* was redissolved in 300 ml. of buffered medium.

Removal of Nucleic Acids—3 ml. of 1 per cent protamine sulfate solution were added drop by drop to a constantly stirring solution. The solution was allowed to stand for 1 hour and then centrifuged at 10,000 × *g* for 10 minutes to give Supernatant II. The precipitate was discarded.

Ammonium Sulfate Fractionation I—To 300 ml. of Supernatant II, solid ammonium sulfate was added to give 20 per cent saturation. After 3 hours the precipitate was removed (to give 0 to 20 per cent ammonium SO_4 Fraction I) and the supernatant fluid was brought to 40 per cent saturation to give Fraction II (20 to 40 per cent saturation). This process was repeated to give fractions precipitated at 60, 80, and 100 per cent saturation. Each fraction was dissolved in buffer medium (to contain 10 mg./ml. estimated by turbidity) and dialyzed for 24 hours against 1000 ml. of the buffer medium with three changes. Each of the fractions so obtained was tested for its activity in the incorporation of amino acid into microsomal protein. The results of this study are given in Table IV.

Preparation of Supernatant Nucleic Acids—A large amount of pH 5 enzymes was precipitated as above from the normal animal livers. The precipitate was then treated with 0.5 per cent NaCl at 50° and the undissolved material was removed by centrifugation. The supernatant fluid was cooled to 0°, and previously cooled (−10°) 95 per cent ethanol was added to precipitate the nucleic acids. The precipitate obtained was dissolved in cold distilled water and dialyzed for 24 hours against distilled water to remove any traces of NaCl. The concentration of nucleic acid in this solution was approximately 5.0 mg./ml. This solution was stored frozen at −20° and used as the source of supernatant nucleic acids.

Distribution of Radioactive-B_{12} in ASF of pH 5 Enzymes—As a large amount of radioactivity was found in pH 5 enzymes, the distribution of B_{12} between the various ASF of this enzyme was investigated. For this purpose, 6.0 μc. of Co^{60}-B_{12} was injected

TABLE III

*Incorporation of Co^{58}-B_{12} by enzyme preparations**

Enzyme preparation	B_{12} status of rats			
	+B_{12}		−B_{12}	
	c.p.m./mg. of protein		c.p.m./mg. of protein	
	pH 5 enzyme	microsomes	pH 5 enzyme	microsomes
Microsomes and supernatant (10.0 mg. of protein in 5 ml.)	151	241	112	203
Microsomes alone (10.0 mg. of protein in 5 ml.)		268		216
Supernatant alone (10.0 mg. of protein in 5 ml.)	132		88	

* Incubation mixture contained 1.0 μmoles FDP, 0.5 μmole ATP, and 0.25 μmole GTP and Co^{58}-B_{12} (60 mμg.). These values are for two rats run in duplicate. There was very good agreement between replicates.

in three doses of 2 μc. each at 8 hour intervals into a normal 250 gm. rat. The rat was sacrificed 8 hours after the last injection. The pH 5 enzymes were prepared and fractionated as described previously. The activity of the various ASF is given in Table V.

Effect of a Vitamin B_{12} Antagonist on Incorporation of Radioactive Amino Acid into Microsomal Protein—In this study, incorporation of radioactive amino acid into microsomal protein was studied by using the pH 5 enzymes plus the microsome system in the presence of a B_{12} antagonist. In a few of the incubations pH 5 enzymes were replaced by 20 to 60 per cent ASF either in the presence or absence of B_{12} antagonist and also in the presence of 4 mg. of 20 to 60 fraction. The results of these studies are given in Table VI. Studies on the effect of a B_{12} antagonist on pyrophosphate exchange are given in Table VII.

TABLE V

Distribution of radioactive B_{12} in ammonium SO_4 fractions

Fractions	Total c.p.m.	c.p.m./mg. of protein
105,000 × g supernatant	88,600	
pH 5 enzymes	67,490	
Activity after nucleic acid separation	62,000	
0–20 per cent ASF	Nil	
20–40 per cent ASF	6,800	2,130
40–60 per cent ASF	51,300	17,250
60–80 per cent ASF	Nil	
80–100 per cent ASF	Nil	

RESULTS AND DISCUSSION

The results of the experiments on the activity of the pH 5 enzymes from B_{12}-deficient and B_{12}-normal rats are summarized in Table I. These data clearly show a lower incorporation of 2-C^{14}-alanine into protein in the *in vitro* system consisting of microsomes + pH 5 enzymes derived from the liver of B_{12}-deficient rats. As with the crude microsome + supernatant system (4) here also the addition of vitamin B_{12} partially restored the amino acid incorporating ability of the deficient system. These data again confirm the requirement for pH 5 enzymes as found by Keller and Zamecnik (8). No effect of addition of vitamin B_{12} was found on incorporation of alanine by the microsomes alone or by pH 5 enzymes alone. However, when microsomes from B_{12}-deficient animals were incubated with the addition of pH 5 enzymes from B_{12}-normal animals, the incorporation activity was raised almost to that of the complete system from the normal animals. This indicates that the components affected by the absence of B_{12} from the diet were associated with pH 5 enzymes. As would be expected if this were the case, the pH 5 enzymes from the deficient animal were less active in increasing the incorporation activity of the norma

TABLE IV

Incorporation of 2-C^{14}-alanine into protein by microsome preparations supplemented with pH 5 enzymes and ASF

System used	c.p.m./mg. of protein
1. Complete system*	93
2. Complete system minus microsomes	32
3. Complete system minus pH 5 enzymes	21
4. 0.3 ml. microsome + 0.5 mg. nucleic acid + 2 mg. ASF (0–20 per cent)	24
5. 0.3 ml. microsome + 0.5 mg. nucleic acid + 2 mg. ASF (20–40 per cent)	67
6. 0.3 ml. microsome + 0.5 mg. nucleic acid + 2 mg. ASF (40–60 per cent)	154
7. 0.3 ml. microsome + 0.5 mg. nucleic acid + 2 mg. ASF (60–80 per cent)	28
8. 0.3 ml. microsome + 0.5 mg. nucleic acid + 2 mg. ASF (80–100 per cent)	20
9. 0.3 ml. microsome + 0.5 mg. nucleic acid + 1 mg. ASF (20–40 per cent) + 1 mg. ASF (40–60 per cent)	216
10. 0.3 ml. microsome + 0.5 mg. nucleic acid + 0.4 mg. of each of the 5 ASF	138
11. 0.3 ml. microsome + 0.5 mg. nucleic acid + 1 mg. ASF (0–20 per cent) + 1 mg. ASF (80–100 per cent)	26
12. 0.3 ml. microsome + 0.5 mg. nucleic acid + 1 mg. ASF (0–20 per cent) + 1 mg. ASF (60–80 per cent)	22
13. 0.3 ml. microsome + 0.5 mg. nucleic acid + 1 mg. ASF (60–80 per cent) + 1 mg. ASF (80–100 per cent)	20
14. 0.3 ml. microsome + 0.5 mg. nucleic acid + 0.66 mg. ASF (0–20 per cent; 60–80 per cent; 80–100 per cent) each	30
15. 0.3 ml. microsome + 0.5 mg. nucleic acid + 0.66 mg. ASF (20–40 per cent; 40–60 per cent; 60–80 per cent) each	166
16. 0.3 ml. microsome + 0.5 mg. nucleic acid + 0.66 mg. ASF (0–20 per cent; 40–60 per cent; 60–80 per cent) each	108

* Complete system contained 0.3 ml. microsomes, 0.2 ml. pH 5 enzymes, 0.25 μmole of 2-C^{14}-alanine, 10 μmoles phosphoenol pyruvate, 0.02 mg. pyruvate kinase, 0.25 μmole GTP, 0.5 μmole ATP. The incubations 4 to 16 contained the same amount of 2-C^{14}-alanine, phosphoenol pyruvate, pyruvate kinase, ATP, and GTP. The final volume of the incubation mixture was made to 1.0 ml. with 0.15 M KCl.

TABLE VI

Incorporation of 2-C^{14}-alanine into microsomal protein by pH 5 enzymes and ASF and its inhibition by B_{12} antagonist

System used	c.p.m./mg. of protein
1. Complete system*	98
2. Complete system minus microsomes	34
3. Complete system minus pH 5 enzymes	24
4. Complete system plus anti-B_{12} (125 μg.)	76
5. Complete system plus anti-B_{12} (250 μg.)	52
6. 0.3 ml. microsome + 0.5 mg. nucleic acid + 1 mg. (20–40 per cent; 40–60 per cent) ASF each	216
7. 0.3 ml. microsome + 0.5 mg. nucleic acid + 1 mg. (20–40 per cent; 40–60 per cent) ASF each + anti-B_{12} (125 μg.)	156
8. 0.3 ml. microsome + 0.5 mg. nucleic acid + 1 mg. (20–40 per cent; 40–60 per cent) ASF each + anti-B_{12} (250 μg.)	122
9. 0.3 ml. microsome + 0.5 mg. nucleic acid + 2 mg. ASF (20–40 per cent) + 2 mg. ASF (40–60 per cent) + anti-B_{12} (250 μg.)	193

* Complete system contained 0.3 ml. microsomal preparation, 0.2 ml. pH 5 enzymes, 0.25 μmole 2-C^{14}-alanine, 10 μmoles phosphoenol pyruvate, 0.02 mg. pyruvate kinase, 0.5 μmole ATP, and 0.25 μmole GTP. The incubations 6 to 9 contained the same amount of 2-C^{14}-alanine, phosphoenol pyruvate, pyruvate kinase, ATP, and GTP. The final volume of the incubation mixture was made 1.0 ml. by the addition of 0.15 M KCl.

microsomes as compared to the complete system from normal animals. A reduction in protein synthesis could be obtained by a decrease in the nucleic acid content of the cell as shown by Gale and Folkes (9). However, previous work (10, 11) has failed to indicate, in the case of the rat, chick, or pig, any effect of vitamin B_{12} deficiency on the biosynthesis of either liver DNA or RNA from several different labeled precursors. However, to be certain that the quantity of nucleic acids was not critical, experiments were carried out (see Table I, *Line 9*) in which 2 mg. of the nucleic acids were added to both the normal and deficient preparations. That the added nucleic acids could not restore the incorporation of the amino acid in B_{12}-deficient animals indicated a direct requirement of B_{12} for protein synthesis rather than indirectly through an effect on nucleic acid synthesis.

Dubnoff (12) working with *Escherichia coli* 113-3 requiring vitamin B_{12} has studied the formation of β-galactosidase. He observed a 4-fold increase in the β-galactosidase activity in the presence of vitamin B_{12}. Based on these results he proposed the following hypothesis:

$$\text{Enzyme—S} \xrightleftharpoons{\text{Substrate}} \text{Enzyme} \xrightleftharpoons{B_{12}} \text{Inactive Enzyme}$$
$$\Updownarrow$$
$$\text{Amino Acids}$$

These results are probably better explained by the hypothesis that vitamin B_{12} functions in protein synthesis (Enzyme $\xrightleftharpoons{B_{12}}$ Amino Acids) rather than in the conversion of the inactive enzyme to the active form.

The data in Table II on the subcellular distribution of radioactive vitamin B_{12} in liver indicate that whereas there is a considerable amount of the vitamin in each fraction the concentrations are highest in the microsomes and supernatant. They also indicate that at least some of the activity in the microsomes is readily removed by washing so that the supernatant is, in fact, the chief site. The remaining radioactivity in the microsomes could not be washed out by hot TCA, alcohol, or water, nor could it be dialyzed off. In the supernatant fluid practically all of the B_{12} is precipitated with the pH 5 enzymes.

The *in vitro* incorporation of Co^{60}-B_{12} into microsomes and pH 5 enzymes (Table III) shows that a large amount of radioactivity is incorporated into these fractions. This study demonstrates that this enzyme system is able to incorporate vitamin B_{12} into its functional site. However, much less radioactivity is incorporated *in vitro* than in the whole animal.

The studies carried out on the activity of ASF of the pH 5 enzymes for the incorporation of amino acids into proteins are shown in Table IV. It can be seen that nucleic acids, and the ASF 20 to 40 and 40 to 60 per cent are absolutely essential for the incorporation of alanine into protein. An increase in incorporating activity of a little over 2-fold was obtained when 20 to 60 per cent ASF replaced crude pH 5 enzymes. The 0 to 20 and 60 to 100 per cent ASF were inactive. From the increased incorporation per mg. of enzyme used, a 2-fold purification in the supernatant enzymes needed for amino acid incorporation was obtained by the ammonium sulfate fractionation. These fractions are certainly not pure and presumably contain several enzymes from the pH 5 precipitate, possibly a different Bu

TABLE VII

Incorporation of P^{32}-labeled pyrophosphate into ATP by (40 to 60 per cent ASF of pH 5 enzymes and its inhibition by anti-B_{12}

System	Total counts incorporated into ATP
	c.p.m.
Complete system* with pH 5 enzymes	846
Complete system with pH 5 enzymes + anti-B_{12} (250 μg.)	388
Complete system + excess pH 5 enzyme (4 mg.) + anti-B_{12} (250 μg.)	460
Complete system with ASF (40–60 per cent)	610
Complete system with ASF (40–60 per cent) + anti-B_{12} (250 μg.)	310
Complete system with ASF (40–60 per cent excess, 4 mg.) + anti-B_{12} (250 μg.)	570

* Complete system contained 5 mg. of pH 5 enzymes or 2 mg. of 40 to 60 per cent ASF; 100 μmoles of tris(hydroxymethyl)-aminomethane 7.6; 5 μmoles ATP; 1 μmole of pyrophosphate activity (PP^{32}) pH 7.5 containing 200,000 c.p.m.; 2 μmoles of $MgCl_2$; 0.25 μmole of alanine made to 1 ml. with 0.15 M KCl. Anti-B_{12} was added as anilide of the monocarboxylic acid pH 7.6.

...e for each amino acid. In the present experiments, alanine only was used, but previous work (4) had shown B_{12} to be involved also in the incorporation of phenylalanine and methionine.

The partially purified enzymes obtained by ammonium sulfate fractionation may be similar to the highly purified tryptophan activating enzyme obtained by Davie *et al.* (13) from beef pancreas. It is probable that the 20 to 40 and 40 to 60 per cent ASF of the pH 5 enzymes contain many different enzymes activating different individual amino acids.

The distribution of the radioactive B_{12} of the supernatant pH 5 enzymes among the various ASF is given in Table V. It can be seen that the radioactivity is entirely in those fractions which are required for amino acid incorporation (Table IV), most of the Co^{60}-B_{12} appearing in the 40 to 60 per cent fraction.

Smith *et al.* (14) have prepared a number of substituted carboxamide derivatives of vitamin B_{12} which have shown B_{12} antagonism in bacteria. One of these, the anilide of the monocarboxylic acid, has been used in the inhibition studies reported here. It can be seen from Table VI that this compound markedly inhibits the incorporation of the amino acid alanine into protein by the microsome + pH 5 enzyme system (Experiment 1 *versus* 4 and 5) and also by the microsome + 20 to 40 and 40 to 60 per cent ASF of the pH 5 enzymes (Experiment 6 *versus* 7 and 8). It can also be seen (Experiment 8 *versus* 9) that doubling the amount of 20 to 40 and 40 to 60 per cent ASF of the pH 5 enzymes (the B_{12} part) reverses this inhibition. This seems to strongly support the previous experiments indicating ...irect role of vitamin B_{12} in amino acid incorporation into ...otein.

An important advance in the understanding of the mechanism of protein synthesis was the finding by Hoagland *et al.* (15, 16) of an ATP amino acid activation step linked with pyrophosphate exchange. DeMoss and Novelli (17) have also demonstrated such an amino acid activating system in microorganisms. It appeared conceivable that B_{12} might function in this initial activation step in view of its presence in the active part of the pH 5 enzymes. Smith (18) has suggested that the labile amide groups of the B_{12} molecule are the functional site and the experiments already cited indicate that the changing of these groups results in an antagonist which inhibits our enzyme system. It is proposed then that vitamin B_{12} might serve via the carboxamide groups for transfer of the amino acid from ATP to supernatant RNA. Preliminary studies with C^{14}-alanine, ATP and the 20 to 60 per cent ASF indicate the formation of bound amino acid. Further purification of the B_{12} enzymes is in progress and it is hoped that this will further elucidate the pathway and role of B_{12} in amino activation. It is perhaps relevant here to refer to the enzyme recently found in liver which catalyzes the exchange of ammonia from protein amide groups with aliphatic amines including the ε-amino group of lysine (19).

Another approach to the study of the site of action of B_{12} in protein synthesis was the use of P^{32}-labeled pyrophosphate and the study of its exchange with ATP in the presence of amino acid and the "B_{12} enzyme" part of the pH 5 enzymes. In these experiments also the effect of the B_{12} antagonist was observed. The results given in Table VII show that this exchange is catalyzed by the crude B_{12} enzymes and is markedly inhibited by the antagonist and that this inhibition is reversible by the addition of more B_{12}-containing enzymes. These experiments suggest an activation reaction for amino acids as:

$$\text{amino acids} + \text{ATP} \xrightleftharpoons{B_{12}\ \text{enzyme}} \sim \text{amino acids} + \text{pyrophosphate activity } (P^{32})$$

The role of vitamin B_{12} in the mechanism of this activation reaction is being studied and will be reported subsequently.

SUMMARY

1. The incorporation of amino acids into protein *in vitro* has been studied within the system prepared from microsomes and the "pH 5 enzyme" fraction of rat liver.

2. A reduced incorporation of amino acid into protein was observed in pH 5 enzyme microsomal preparations obtained from animals deficient in vitamin B_{12}. This reduction could be partially overcome by the addition of crystalline B_{12} and completely by the addition of pH 5 enzyme preparation from B_{12}-normal animals.

3. A major portion of the B_{12} activity was found in the pH 5 enzyme fraction prepared from the liver of a normal rat injected with Co^{60}-B_{12}.

4. Incubation of radioactive B_{12} with the pH 5 enzyme-microsome system showed that a large amount of B_{12} is incorporated *in vitro* into microsomes and into pH 5 enzymes.

5. Ammonium sulfate fractionation of the pH 5 enzyme preparation led to a 2-fold increase of amino acid incorporation into protein.

6. A large amount of radioactive B_{12} was found in the 40 to 60 per cent ammonium sulfate fraction and a smaller amount in the 20 to 40 per cent fraction when these fractions were obtained from pH 5 enzymes isolated from the liver of a rat given Co^{60}-B_{12}.

7. 40 per cent inhibition of the incorporation of amino acid was obtained when a B_{12} antagonist was added to the pH 5 enzyme microsome system. This inhibition could be partially restored by the addition of excess pH 5 enzymes and almost completely by the addition of 20 to 40 and 40 to 60 per cent ammonium sulfate fractions (B_{12} enzyme fraction of pH 5 enzyme).

8. The 40 to 60 per cent ammonium sulfate fraction enzyme was able to incorporate pyrophosphate activity (PP^{32}) into adenosine triphosphate when incubated with adenosine triphosphates, amino acids, enzyme, and pyrophosphate activity (PP^{32}). This exchange could be inhibited by the addition of the B_{12} antagonist.

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VITAMIN B_{12} AND RELATED COMPOUNDS[1,2]

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This review attempts to analyze current and recent literature on the chemistry and biochemistry of vitamin B_{12} and related derivatives. After a brief introduction, the biogenesis, microbiological degradation and chemistry of these compounds and their role in enzymatic reactions are discussed. In recent years several reviews (1–14) have appeared which provide an excellent account of the broader aspects of vitamin B_{12}.

Introduction

Research in the field of vitamin B_{12} has been in a state of continuous and rapid development ever since this compound was isolated in 1948 (15, 16, 17). These studies culminated in the elucidation of the structure of cyanocobalamin (Figure 1, L = CN and R = H) by Hodgkin and co-workers (1, 18, 19) and in the recent discovery (20) of a cobalt-carbon bond in the structure of one of Barker's B_{12} coenzymes (Figure 1, L = 5′-deoxyadenosyl and R = H). The isolation of the coenzyme forms of vitamin B_{12} and its derivatives by Barker and his group (21, 22, 23) started a new phase in the understanding of the role of vitamin B_{12} compounds in metabolism.

The elucidation of the structures of CN-B_{12} and 5′-deoxyadenosyl-B_{12} has enriched the chemistry of natural products with a class of structures containing two remarkable features: (*a*) the corrinoid ligand system, and (*b*) a cobalt-carbon bond.

In the corrinoid ligand system (corrin nucleus and cobalt atom) the central cobalt atom is unusually stable. The cobalt atom does not exchange in the Co^{+3} or Co^{+2} state (24) or in the Co^{+1}-corrinoids under the conditions for the synthesis of coenzyme forms (25). The corrin nucleus in CN-B_{12} and 5′-deoxyadenosyl-B_{12} is nonplanar (26), but more nearly planar (26) in the two "incomplete" corrinoids: cobyric acid (27) and 8-aminocobyrinic acid-c-lactam (28). These variations seem to depend on the substituents on the fifth ("lower") and sixth ("upper") coordination side of the cobalt atom. The two sides of the corrin nucleus in all the natural corrinoids are significantly different, both in the geometrical distribution of the substituent groups and espe-

[1] The survey of literature pertaining to this review was concluded in August 1965.

[2] The following abbreviations will be used: CN-B_{12} for α-(5,6-dimethylbenzimidazolyl)-cyanocobamide; OH-B_{12} for α-(5,6-dimethylbenzimidazolyl)-aquo(hydroxo)cobamide; 5′-deoxyadenosyl-B_{12} for α-(5,6-dimethylbenzimidazolyl)-5′-deoxyadenosylcobamide; methyl-B_{12} for α-(5,6-dimethylbenzimidazolyl)-methyl(alkyl)cobamide; B_{12r} for a one-electron reduced form of OH-B_{12} having cobalt in a valence state of 2; B_{12s} for a two-electron reduced form of OH-B_{12} containing monovalent cobalt; corrinoid, as a general name for compounds containing the corrin nucleus.

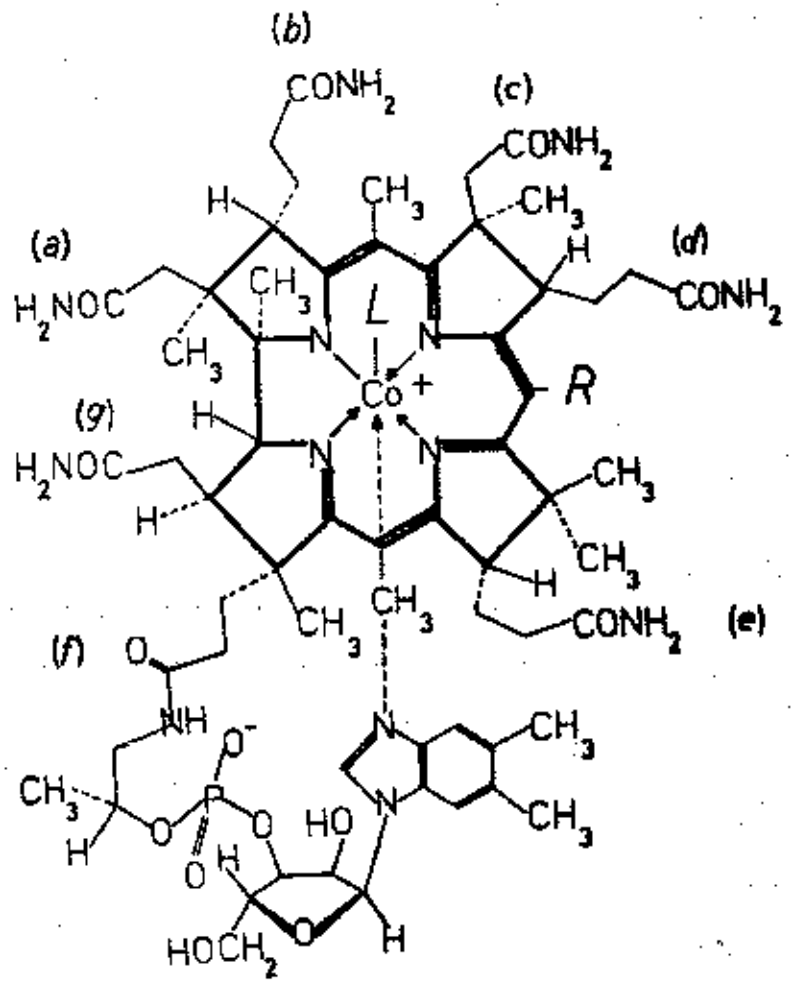


FIG. 1. Structures of cobalamins.

cially in the reactivity of the cobalt atom at the upper and lower coordination positions (26, 29). Studies of the electronic structure of vitamin B_{12} by the molecular orbital method indicated that the corrinoid ligand system should be a good electron acceptor (30).

The second striking feature of the corrinoid coenzymes is the presence of a cobalt-carbon bond. It is the first case of an organometallic compound in living systems. From the magnitude of the angle formed by Co-C_5'—C_1' in 5'-deoxyadenosyl-B_{12}, Lenhert & Hodgkin (20) concluded that this cobalt-carbon bond has a partial ionic character. Considerations of the remarkable differences in the spectra (31–34) and in the polarographic behavior (35, 36) of corrinoids with carbon ligands suggest that changes in the inductive character of the carbon ligand cause changes in electron density of the corrin nucleus. These interactions between axial ligands and the conjugated corrinoid ligand system have been proposed to result from a perpendicular conjugation (34).

The study of model substances may surely offer a deeper understanding of the chemical and biochemical aspects of the corrinoid system. The elegant synthesis of pentamethyl corrin which led to such a model has been described by Eschenmoser (37). Chemical and physical investigations on this model would be of great interest for further knowledge of vitamin B_{12}.

BIOGENESIS OF CORRINOIDS

The corrin moiety.—Vitamin B_{12} is unique among the vitamins in being synthesized probably only by microorganisms (14, 38, 39). An apparent exception to this statement was the finding [Woolley (40, 41, 42)] by indirect evidence that vitamin B_{12} is synthesized by spontaneous mammary tumours in mice. According to more recent observations, rabbits and mice synthesize vitamin B_{12} *de novo* for their own needs (43).

Investigations concerning the biosynthesis of the vitamin B_{12} molecule have shown that the corrin moiety is synthesized from the known precursors of porphyrins (44, 45, 46). These experiments indicated that C^{14}-labelled glycine, δ-aminolevulinic acid and porphobilinogen are incorporated into vitamin B_{12} by bacterial cultures. Bray & Shemin (47, 48) demonstrated with C^{14}-methyl-labelled methionine that six of the eight methyl groups around the macro ring are derived from the methyl group of methionine, probably by a mechanism of C-alkylation, as postulated by Bonnett et al. (49). One of the two *gem* methyl groups on ring C at C-12 arises by decarboxylation of an acetic acid residue originally present (48, 50). Finally, it appears also that the methyl group on ring A at C-1 is derived, like the *meso* carbons, from the δ-carbon of δ-aminolevulinic acid (48, 50). The assumption of Bukin et al. (51, 52) that methylated derivatives of δ-aminolevulinic acid are true precursors of the corrin structure does not agree with the above mentioned results. For these experiments (48) the labelled vitamin B_{12} was synthesized with the aforementioned precursors with growing cultures of an actinomyces (ATCC 11073). It is noteworthy that these fermentations with radioactive precursors labelled only the cobyrinic acid part of vitamin B_{12}. Bykhovskii, Zaitseva & Mantrova (53) reported that resting cells of *Propionibacterium shermanii* could utilize δ-aminolevulinic acid for biosynthesis of vitamin B_{12}.

Mechanisms have been advanced to explain the formation of both uroporphyrinogen III and corrinoids from porphobilinogen (54, 55, 56). The stage at which methylation and decarboxylation of the acetic acid group at ring C occurs is not known. From the position of the methyl groups in vitamin B_{12}, Hodgkin (26) assumed that methylation takes place before ring

closure. It is possible that incorporation of the cobalt atom occurs into an open chain form and that the coordinated cobalt atom favored the ring closure between ring A and D. The cobalt-free macrocycle has not yet been observed. Porra & Ross (57) studied the enzymatic formation of cobalt porphyrins by extracts of *Clostridium tetanomorphum*. The authors suggested that a specific enzyme, cobalt-porphyrin-synthase, catalyzes the incorporation of Co^{++} into tetrapyrrole pigments during biogenesis of vitamin B_{12}.

From cobyrinic acid to cobinamide.—The first part of the biogenesis of corrinoids can be considered complete with the formation of cobyrinic acid. Cultures of *Propionibacterium shermanii*, grown in the presence of cobalt but without the precursor 5,6-dimethylbenzimidazole, produce mainly cobinamide with lesser quantities of cobinic acid mono- to pentaamide and cobyrinic acid di- to hexaamide, including cobyric acid (58, 59). Cobyric acid (cobyrinic acid-*abcdeg*-hexaamide) was also isolated from digested sewage sludge (60, 61) and obtained in crystalline form (27). 5'-Deoxyadenosyl-cobyric acid was found in a mutant of *Nocardia rugosa* (62), in *P. shermanii* (63, 64) and recently in *Clostridium thermoaceticum*, where it constitutes about 50 per cent of the corrinoid content (65). Of the greatest interest is the presence in *Cl. thermoaceticum* of methylcobyric acid and possibly also of the carboxymethylcobyric acid (65, 66). It seems that the above-mentioned cobyrinic acid and cobinic acid derivatives are intermediates in the biosynthesis of vitamin B_{12} by *P. shermanii* (5, 9, 58, 59, 67, 68, 69), by *N. rugosa* mutants (70, 71, 72), by *Methanobacillus omelianskii* (73), and by *Cl. thermoaceticum* (65). DiMarco et al. (70, 71) have isolated mutants of *N. rugosa* some of which introduce the amide group and others the aminopropanol group. Each mutant accumulates a different precursor of vitamin B_{12}, indicating a metabolic block at different stages in biosynthesis of the vitamin.

Bernhauer et al. (64) first isolated cobyrinic acid and cobyrinic acid monoamide beside a small amount of cobinic acid in studies on very young growing cultures of *P. shermanii*. The results obtained at different stages of growth are presented in Figure 3 (64). The reamidation method (74) was used to distinguish between the cobyrinic acid and cobinic acid derivatives. The former derivatives were converted by this method to cobyramide and the latter to cobinamide. Experiments with cell-free extracts of *P. shermanii* demonstrated that cobyrinic acid, cobyrinic acid monoamide and cobinic acid [produced by acid hydrolysis of vitamin B_{12} or cobinamide (64, 74)] cannot be converted into the coenzyme forms, whereas the other acid derivatives yield the coenzyme forms (64). At present it is not possible to say which are the natural forms of the cobyrinic acid, cobyrinic acid monoamide, and cobinic acid. Investigations by Friedrich (69) suggest that cobyrinic acid triamide, cobyrinic acid tetraamide and cobyrinic acid pentaamide—prepared by acid hydrolysis of cobyric acid and obtained in crystalline form—were amidated very rapidly by growing cultures of *P. shermanii*, with accumulation of cobyric acid. *Lactobacillus leichmanii* and *Euglena gracilis* possess enzymes that amidate the free propionic acid groups in the corrinoids (51).

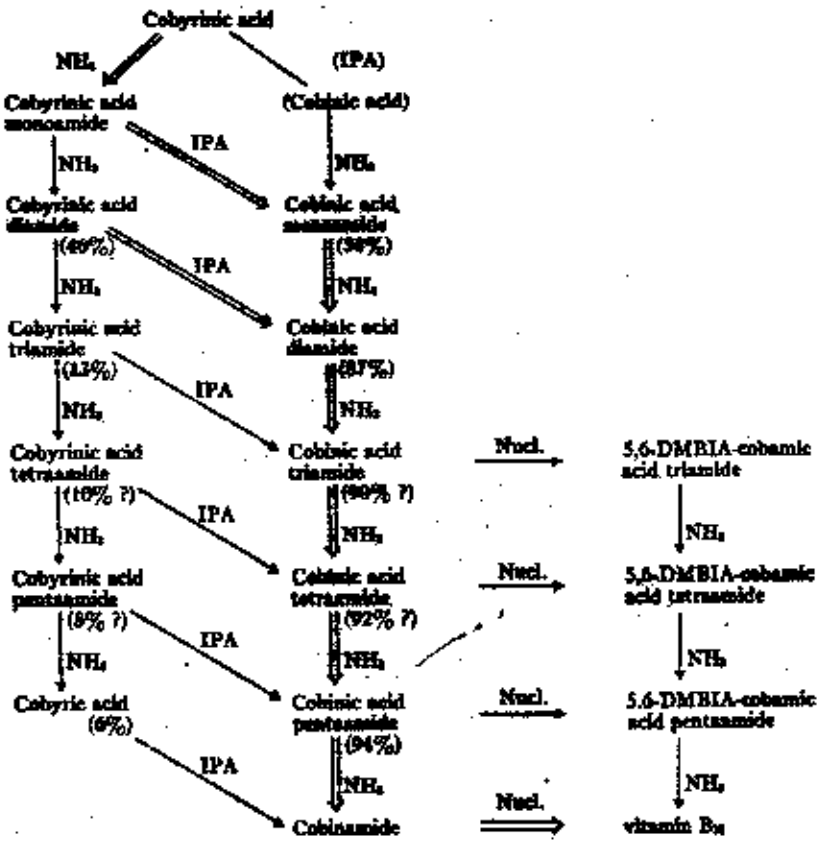


FIG. 2. Supposed pathway of the biogenesis of vitamin B_{12}.

⇒ = main pathway
→ = side pathway
} in the case of *Propionibacterium shermanii*
IPA = incorporation of the isopropanolamine group
NH_3 = amidation of one carboxylic acid group
Nucl. = incorporation of the nucleotide without considering the real pathway
? = estimate

In view of these results and of other considerations it is possible to postulate a biosynthetic pathway from cobyrinic acid to cobinamide (see Fig. 2). Of primary importance for cobinamide biosynthesis seems to be the fact that one acetic acid residue in cobyrinic acid is amidated. The resulting acetamide residue is probably in position *e* and is necessary for the formation of the coenzyme forms if the indicated sequence of amidation on the cobinic acid

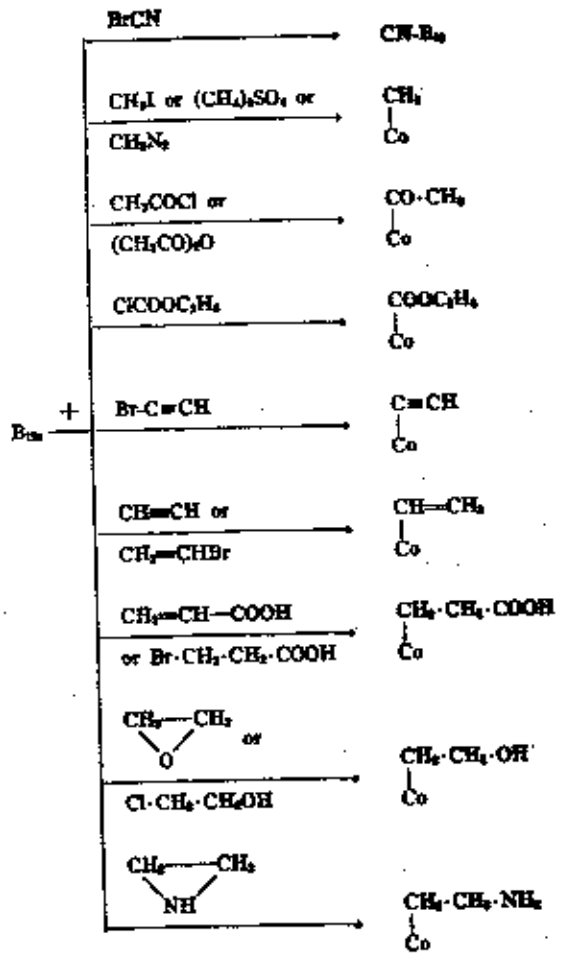


FIG. 3. Examples for the partial synthesis of various alkyl derivatives of vitamin B_{12a}.

main pathway and the supplementary cobyrinic triamide pathway are followed. The next steps are the incorporation of the aminopropanol group in position *f* and amidation of the acetic acid groups in *a* and *g* resulting in the cobinic acid-*acg*-triamide. Further reactions are the amidation of the free propionic acid groups, predominant in the sequence *d*, *b*, and *e*, yielding finally cobinamide. At these last amidation steps, a nucleotide or a part of a nucleotide can be introduced into these cobinic acid derivatives, as shown by the

appearance of the corresponding α-(5,6-dimethylbenzimidazolyl)-cobamic acids, and then amidated to vitamin B_{12}. The aminopropanol group of vitamin B_{12} is derived from L-threonine (75). *Escherichia coli* 113-3 and *P. shermanii* seem to effect decarboxylation prior to incorporation of threonine into cobyric acid (76, 77). The incorporation of aminopropanol and complete amidation leads to cobinamide which is the most common intermediate in the biogenesis of cobamides (see reviews 9, 12, 14).

Biogenesis of the nucleotide part.—In corrinoid-producing microorganisms there have been found some compounds which are probably intermediates along the route from cobinamide to vitamin B_{12} (see reviews 9, 12). Barchietti et al. (78) have isolated from cultures of *N. rugosa* cobinamide phosphate (cobinamide-P) and P_1-cobinamide-P_2-guanosine diphosphate (cobinamide-PPG). Reversed-labeling experiments with [^{32}P]-phosphate (79) indicate that the phosphate group of cobinamide-P and the cobinamide-P moiety of cobinamide-PPG become incorporated into vitamin B_{12} by growing cultures of *N. rugosa*. Experiments in the presence of 5,6-dimethylbenzimidazole or 1-α-D-ribofuranosyl-5,6-dimethylbenzimidazole (α-ribazole) and [^{14}C]-ribose suggested that the α-ribazole is incorporated as such into vitamin B_{12} by this microorganism (80). From these data it was concluded that the cobinamide-P and cobinamide-PPG are intermediates, and the scheme below has been suggested for the biosynthesis of vitamin B_{12}:

$$\text{Cobinamide} + \text{ATP} \rightarrow \text{Cobinamide-P} + \text{ADP}$$
$$\text{Cobinamide-P} + \text{GTP} \rightarrow \text{Cobinamide-PPG} + \text{PP}_i$$
$$\text{Cobinamide-PPG} + \alpha\text{-Ribazole} \rightarrow \text{Vitamin } B_{12} + \text{GMP}$$

The replacement of GMP in this reaction may be similar to the formation of phosphatides (81).

In cultures without 5,6-dimethylbenzimidazole, *P. shermanii* produces cobinamide-P (64), cobinamide-PPG (64, 82) and, in addition to these, cobinic acid-*abcdg*-pentaamide phosphate and P_1-cobinic acid-*abcdg*-pentaamide-P_2-guanosine-diphosphate (64). All these compounds acted as precursors of vitamin B_{12} in this microorganism. Utilization of [^{32}P]-cobinamide-P has shown that it is transferred into [^{32}P]-vitamin B_{12} by growing *P. shermanii* (83). The labelled cobinamide-P was synthesized by condensing natural cobinamide with [^{32}P]-β-cyanoethylphosphate and N,N'-dicyclohexylcarbodiimide yielding [^{32}P]-β-cyanoethylcobinamide phosphate, which gave [^{32}P]-cobinamide-P upon alkaline hydrolysis (83, 84). Cobinamide-PPG and P_1-cobinamide-P_2-adenosine diphosphate (cobinamide-PPA) was synthesized by starting with cobinamide-P (85). Cell-free extracts from *P. shermanii* converted cobinamide, cobinamide-P, cobinamide-PPG, cobinamide-PPA, and cobinamide-phosphoribose in the presence of the precursors 5,6-dimethylbenzimidazole, α-ribazole, or α-ribazole-3'-phosphate into vitamin B_{12} coenzyme in nearly the same yield (83).

Friedmann & Harris (86, 87) obtained evidence for an enzyme in *P. shermanii* which catalyzes a reaction between 5,6-dimethylbenzimidazole (5,6-

DMBIA) and nicotinic acid mononucleotide (β-N_aMN^+) to form α-ribazole-5'-phosphate. The latter product is readily dephosphorylated by an endogenous phosphatase:

$$\text{5,6-DMBIA} + \beta\text{-}N_aMN^+ \rightarrow \alpha\text{-ribazole-5'-P} + \text{nicotinate} + H^+ \rightarrow \alpha\text{-ribazole-5'-P} + H_2O \rightarrow \alpha\text{-ribazole} + P_i$$

It was shown that a similar reaction takes place with a number of 5-substituted benzimidazoles, but not with 2-methylbenzimidazole. However, the α-configuration of the resulting nucleosides has been demonstrated only for nucleosides of 5,6-dimethylbenzimidazole and benzimidazole (88). The enzyme reacts as a single displacement trans-N-glycosidase in the formation of an N-α-glycosidic bond. The role of this enzyme in guided biosynthesis of vitamin-B_{12} analogues was discussed (88).

Studies of Bernhauer and co-workers on the biosynthesis of vitamin B_{12} by a wild-type of *E. coli* (89, 90) and with *P. shermanii* (91) have suggested the following sequence of reactions (see review 59):

$$\text{Cobinamide-P} \rightarrow \text{Cobinamide-phosphoribose-1'-phosphate} \xrightarrow{+\text{5,6-DMBIA}} \text{vitamin } B_{12} + P_i$$

This hypothesis agrees with the findings that cobinamide-phosphoribose can be utilized by *E. coli* for vitamin B_{12} synthesis; that 5,6-dimethylbenzimidazole is more efficiently utilized in the conversion of cobinamide to vitamin B_{12} than either α-ribazole or α-ribazole-3'-phosphate, and, finally, that *P. shermanii* cultures are able to convert the purine- and the benzimidazole cobamides to vitamin B_{12} when they are incubated in the presence of 5,6 dimethylbenzimidazole. However, further studies by Renz (92) on the latter point have shown that the nucleosides in these compounds are apparently replaced by preformed α-ribazole. [^{32}P]-Adenylcobamide is transformed by *P. shermanii* into [^{32}P]-vitamin B_{12} in the presence of 5,6-dimethylbenzimidazole. The vitamin B_{12} formed from [^{14}C]-benzimidazolyl-cobamide, synthesized from cobinamide, benzimidazole, and u-[^{14}C]-D-glucose using *P. shermanii*, was not radioactive.

The rates of growth of *E. coli* 113-3 obtained in the presence of supposed intermediates in B_{12}-biosynthesis are summarized in Table 1 (33). The data obtained by the investigations with *N. rugosa*, *P. shermanii*, and *E. coli* 133-3 suggest that cobinamide-P and α-ribazole are intermediates in the biosynthesis of vitamin B_{12} and probably of its base analogues. It cannot yet be decided whether cobinamide-PPG is an immediate precursor of the cobamides in *N. rugosa* and *P. shermanii*. In *E. coli* 113-3 it seems likely that cobinamide-PPG and cobinamide-phosphoribose are depots rather than true intermediates.

Some ideas on the biosynthesis of the benzimidazole moiety in vitamin B_{12} have been published (59).

Biosyntheses of 5'-deoxyadenosylcorrinoids.—The enzymatic conversion of CN-B_{12} or OH-B_{12} to 5'-deoxyadenosyl-B_{12} takes place in cell-free systems de-

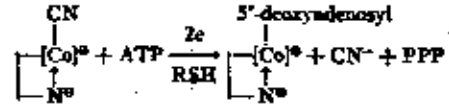
TABLE I

BIOLOGICAL ACTIVITY OF CORRINOIDS FOR *E. coli* 113-3 (ATCC 9637)

Corrinoid tested*	Time of incubation in hours		
	10	16	24
CN-B_{12}	100	100	100
Cobinamide	72	82	98
Cobinamide-P	87	95	101
Cobinamide-PPG	38	42	76
Cobinamide-PPA	38	43	74
Cobinamidephosphoribose	43	61	86

* Maximum activity is indicated by 100.

rived from *P. shermanii* (93, 94) and *Cl. tetanomorphum* (95–98). The enzyme system requires ATP, NADH, FAD, reduced glutathione or mercaptoethanol. A purified enzyme preparation, which had been enriched 337 times, had a requirement for ATP, NADH, FAD, reduced glutathione, Mn^{2+} and K^+ (94). Studies with uniformly labelled [^{14}C]-ATP have shown that ATP serves as a substrate, transferring a 5'deoxyadenosyl moiety in order to form 5'-deoxyadenosyl-B_{12} (95). Remarkable in this reaction is the unique cleavage of ATP resulting in the formation of inorganic tripolyphosphate (98, 99). However, the chemical nature of the 5'-deoxyadenosyl moiety is unknown. Studies on the release of cyanide from CN-B_{12} and the spectral changes during the enzymatic synthesis of 5'-deoxyadenosyl-B_{12} have indicated that a reduced form of vitamin B_{12} is not an obligatory intermediate in the synthesis itself (97). B_{12a} can serve as a substrate, but there is still a requirement for reducing agents (98). From these results, Peterkofsky & Weissbach (99) have concluded that the conversion of CN-B_{12} to 5'deoxyadenosyl-B_{12} occurs in a single concerted reaction in which the bound cyanide anion is replaced by the adenosyl moiety of ATP. The transfer reaction requires two electrons and takes the following course:

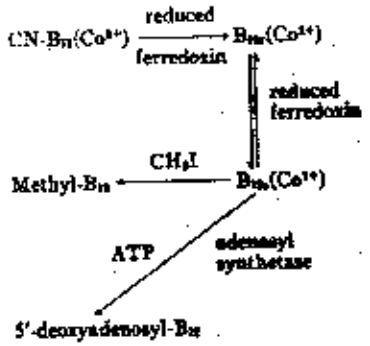


Experiments with the 5'-deoxyadenosyl-B_{12}-synthesizing system from *Cl. tetanomorphum* indicated a stepwise mechanism in which B_{12a} is the reactant with ATP (100).

$$\text{OH-}B_{12} \rightarrow B_{12r} \rightarrow B_{12a}$$
$$B_{12a} + \text{ATP} \rightarrow \text{5'-deoxyadenosyl-}B_{12}$$

For the overall reaction NADH and FAD could serve as reductants; however, reduced lipoic acid was a much better reductant. Evidence for the step-

wise reaction was provided by the reaction of chemically prepared B_{12a} with a purified adenosylating enzyme: this enzyme has lost its ability to synthesize 5′-deoxyadenosyl-B_{12} from OH-B_{12}, but not to transfer the adenosyl group of ATP to B_{12a}. Weisbach, Brot & Lovenberg (101) presented evidence that reduced ferredoxin (102, 103) can mediate reduction of cobamide compounds and functions in the enzymatic conversion of CN-B_{12} into 5′-deoxyadenosyl-B_{12}. The reduction of CN-B_{12} by reduced ferredoxin seemed to occur non-enzymatically: the resulting reduced corrinoid reacts either with methyl iodide to produce methyl-B_{12} or, in the presence of adenosyl synthetase from *Cl. tetanomorphum*, with ATP to yield 5′-deoxyadenosyl-B_{12}. These reactions are summarized below.



Between pH 7 and 11, B_{12r} decomposes water to yield hydrogen and B_{12a} (104). However, in the presence of an electrophilic agent it is possible to alkylate the B_{12r} as it is formed in this equilibrium, a fact which provides a useful way to detect the transient formation of B_{12r} and was earlier demonstrated by non-enzymatic transfer reactions (9, 105, 106) and by enzymatic studies (107).

There is increasing evidence that two electron reduced corrinoids are the active substrates for the biogenesis of the cobalt-carbon bond in the corrinoid coenzyme forms and that the electrophilic substitution of these reduced corrinoids is an essential feature of these reactions. An enzymatic system which catafyzes the specific two electron reductions has not been demonstrated yet. It seems likely that many naturally occurring reductants, such as reduced flavin, glutathione, reduced lipoic acid, or reduced ferredoxin can effect corrinoid reduction *in vitro*. The observations by Beck & Hardy (108) suggested that OH-B_{12} is converted on the ribosomes from *Lactobacillus leichmanii* to 5′-deoxyadenosyl-B_{12} by enzymes requiring dihydrolipoic acid or β-mercaptoethanol and ATP.

Recently, several reviews (9, 12, 14, 39) have discussed the occurrence, isolation, and biosynthesis *in vivo* and *in vitro* of 5′-deoxyadenosylcorrinoids. The major corrinoids occurring in nature apparently exist in this coenzyme form. The 5′-deoxyadenosyl group is presumably introduced soon after the biosynthesis of the corrin ring, probably at the stages of cobinic acid monoamide or cobyrinic acid diamide (64). In addition to these findings, Lexius & Barker (73) have isolated ten different corrinoid compounds from extracts of pure cultures of *Methanobacillus omelianskii*. The two most abundant compounds were identified as α-(5-hydroxybenzimidazolyl)-cobamide and α-(5-hydroxybenzimidazolyl)-5′-deoxyadenosylcobamide.

Isolation of methylcorrinoids.—The first isolation of methylcobalamin (methyl-B_{12}) from biological material was published by Lindstrand & Stahlberg (109, 110). It was found in human plasma (109), in calf liver, *E. coli*, and *Streptomyces rimosus* (110). Irion & Ljungdahl (65) have identified eleven corrinoid compounds from acetone extracts of *Cl. thermoaceticum*. The main products are the 5′-deoxyadenosyl derivatives of cobyric acid and α-(5-methoxybenzimidazolyl)-cobamide. Both corrinoids also are present in minute amounts as their methyl derivatives. Other corrinoids present in small amounts are the 5′-deoxyadenosyl derivatives of cobinamide, cobinamide-phosphate and most likely the di- to pentaamides of cobyrinic acid. The natural occurrence of methylcorrinoids can be understood in terms of the corrinoid-dependent enzymatic synthesis of methionine, methane, and acetate (see below).

Microbiological Degradation

CN-B_{12} is converted by growing cultures of *Aerobacter aerogenes* mainly to two yellow pigments (111). One of these pigments is active against pernicious anemia, though it has negligible microbiological activity (112). The yield of this pigment is considerably higher under anaerobic conditions. During an investigation on catabolism of CN-B_{12} Pfiffner et al. (113–116) found several bacteria that formed new colored products from the vitamin B_{12}. Among the bacteria studied, *Pseudomonas rubescens* produces several yellow pigment fractions in the highest yields under anaerobic conditions and in the dark. Studies with [^{58}Co]-, [^{14}C]- or [^{32}P]-labelled CN-B_{12} have shown, in agreement with spectral data, that the axial ligands in the yellow pigments are the same as those in CN-B_{12} (115, 116). These observations make it probable that in the yellow pigments the conjugation of double bonds has been interrupted either by reduction or by addition of one or more double bonds (116). The physical properties of these pigments are similar to those of by-products obtained by photolysis of 5′-deoxyadenosyl-B_{12} (117) and of diaquocobinamide (118), and, as the main product from methylcobyrinic acid heptaethylester or the corresponding hepta *i*-alcohol by photolysis in ethanol (119, 120). In addition, similar yellow products have been obtained by the reaction of OH-B_{12} with thiols (9, 121) and as by-products from the reduction of corrinoids with sodium borohydride or with zinc and acetic acid.

A relationship between the pigments isolated from microorganisms and the yellow "methylcorphyria" arising from a mixture of γ-picoline and OH-B_{12} (122) was discussed. However, this product is probably a tautomeric form (form B) of vitamin B_{12} (120, 123) in which C-10 has become a tetrahedral methylene group. No conversion to a yellow pigment takes place by the

action of γ-picoline on aquo-dehydrovitamin B_{12} [8-amino-α-(5,6-dimethylbenzimidazolyl)-aquocobamic acid-cibdeg-pentaamide-c-lactam], which lacks a proton in position C-8 on ring B (129). Therefore, a substitution on the chromophore in vitamin B_{12} is extremely unlikely.

Chemical Reactions

The elucidation of the structure of the 5'-deoxyadenosyl-B_{12} by X-ray crystallography (20) was soon followed by a partial synthesis of this compound (124–127) involving a two electron reduction of vitamin B_{12} to B_{12r}, reaction of the latter with 2',3'-isopropylidene-5'-tosyladenosine, and hydrolytic removal of the isopropylidene group. The discovery of various methods to synthesize corrinoids with cobalt-carbon or with cobalt-sulphur bonds has made available wide varieties of corrinoids for the study of their chemical and biological behavior (see reviews 5, 6, 9, 14).

Reductions of corrinoids.—A variety of reducing agents, including catalytic hydrogen, sodium borohydride, zinc dust in aqueous ammonium chloride or 0.1 N acetic acid, hypophosphorous acid, chromous acetate, and thiols (see reviews 6, 9), has been used for reduction of corrinoids. Continued reduction with most of these agents leads through yellow pigments to colorless compounds. In certain cases, intermediate brown and grey-green reduction stages also may be distinguished. The stepwise reduction of CN-B_{12} by catalytic hydrogenation (128, 129) or with chromous acetate at pH 5 (130) yields B_{12r}, an orange-brown product. Reduction with chromous acetate at pH 9.5 (131), with zinc dust in aqueous ammonium chloride (132) or with sodium borohydride, changes the color from red to orange-brown and finally to grey-green. This grey-green product has been called B_{12a} (122). It is now accepted generally that B_{12r} is a one-equivalent reduction product of CN-B_{12} containing Co^{2+}, and that B_{12a} is a two-equivalent reduction product of CN-B_{12} containing Co^{1+}. These assignments agree with polarographic studies (134), with the electron spin resonance spectrum of B_{12r} (135), with quantitative hydrogenation of OH-B_{12} to B_{12r} (136), with amperometric titration (104), with controlled potential electrolysis at a mercury cathode to produce B_{12a} from CN-B_{12} (104, 137) and finally with the observation that equimolar mixtures of OH-B_{12} and B_{12a} give 2 equivalents of B_{12r} (122). B_{12a} slowly decomposes water to yield hydrogen and B_{12r} (104). Both reduction products, B_{12r} and B_{12a}, are rapidly oxidized to OH-B_{12} by air. Controlled electrooxidation of B_{12a} gives OH-B_{12} in two one-electron steps (104, 137). The unstable reduction product, B_{12a}, once thought to be a cobalt hydride (hydridocobalamin), can be formulated as an equilibrium mixture (122, 126, 127, 139, 140):

$$Co^{1+} \rightleftharpoons Co^{3+}H^{\ominus}$$

Experiments in deuterium-rich medium suggest that B_{12a} can best be formulated as a compound of the $Co^{3+}H^{+}$ form (138).

Synthesis of cobalt-carbon bond.—A wide range of alkyl halides, dialkylsulfates, trialkylphosphates or *p*-toluenesulfonic esters reacts very rapidly at room temperature with B_{12a} or with other two-electron reduced corrinoids; and crystalline products have been isolated containing the alkyl group linked directly to the cobalt atom (32, 124–127, 140–145). Acylating agents react similarly yielding acylcorrinoids. On treatment with diazomethane, S-adenosylmethionine or S-methyl-methionine, B_{12a} gives methyl-B_{12} (126, 127, 146). Bulky alkyl groups, e.g., *t*-butyl bromide, do not react with B_{12a}.

Addition reactions occur on treatment of B_{12a} with acetylenes, activated olefines, ethylene oxide, tetrahydrofuran (126, 127, 140), and ethylene imine (147). The synthesis of alkylcorrinoids from thiol corrinoid complexes is discussed below.

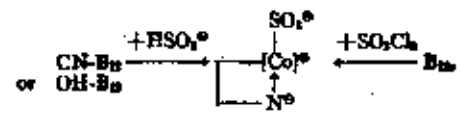
Alkylation of B_{12a} with 2',3'-isopropylidene-5'-tosyladenosine and hydrolytic removal of the isopropylidene group, leads to 5'-deoxyadenosyl-B_{12} (126, 127). Several analogues of 5'-deoxyadenosyl-B_{12} have been synthesized by substituting other 5'-deoxynucleosides for 5'-deoxyadenosyl group (126, 127, 148, 149).

The alkylcorrinoids obtained by partial syntheses have been assigned structures on the basis of their relationship to the natural 5'-deoxyadenosyl-B_{12} as indicated by spectrophotometric and chemical evidence. Elementary analyses on most of these products have not been performed yet.

Remarkable studies by Yamada et al. (150), have shown that B_{12a}-like corrinoids are obtained by alkali treatment of CN-B_{12}, which then reacts with methyliodide yielding methylcorrinoids.

These electrophilic substitution and addition reactions are summarized in Figure 3. (Co^{2+})-corrinoids also can be converted directly into alkylcorrinoids by nucleophilic substitution with Grignard reagents or lithium alkyls, when compounds are used which are soluble in inert solvents (119, 120). For example, treatment of heptaethyl cobyrinate with an excess of methylmagnesium iodide in tetrahydrofuran/ether yields the methyl derivative of the corresponding tertiary alcohol.

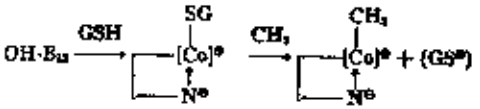
Synthesis of cobalt-sulphur bond.—Treatment of cyano- or aquocorrinoids with bisulfite, sulfurous acid or dithionite, yields products which are similar, in their spectroscopic properties, to the alkylcorrinoids (122, 151–154). These products were also obtained, e.g., by the reaction of B_{12a} or (Co^{1+})-cobinamide with sulfuryl chloride (153). From the physical and chemical properties, both the crystalline B_{12} and cobinamide derivatives can be formulated as cobalamin-sulfonate and cobinamide-sulfonate containing Co^{3+} and a Co-S bond (153).



Cobalamin-sulfonate

p-Toluenesulfonylcobinamide and benzenesulfonylcobinamide (153), and *p*-toluenesulfonyl-B_{12} (155) were prepared in the same way.

An interesting synthesis of alkyl-B_{12} has been found in the reaction of OH-B_{12} with thiols (120). When OH-B_{12} was treated with an excess of glutathione (GSH) in aqueous solution at pH 2–8, a violet product was formed (120, 156) which yields alkyl-B_{12} in the presence of alkylating agents (120). The violet product is stable only in solution and in the presence of excess GSH, but the electrophoretic behavior indicates a 1:1 molar complex of GSH and cobalamin.



It has been reported (9, 155, 157) that GSH and other thiols, including sodium hydrogensulfide, will react with OH-B_{12} to first form violet- and then brown-colored complexes, and that under aerobic conditions the thiol component is oxidized to the disulfide (158, 159). Dolphin & Johnson have also shown that these violet cobalamin-thiol complexes can be converted into alkyl-B_{12} with alkylating agents (155). This fact modified their earlier views on the alkylation mechanism of the brown-colored thiol complexes (157).

Photolysis.—According to their behavior on photolysis the corrinoids can be subdivided into three groups: (*a*) those stable to prolonged photolysis, both in the presence and absence of oxygen, as the aquo-, sulphato-, and thiocyanato-B_{12} (155, 160); (*b*) those undergoing photoaquation leading to an aquocobaltic complex, as in the case of CN-B_{12}, ammonia-B_{12} (160), monoacetato-monoaquo-cobinamide, and monochloro-monoaquo-cobinamide (151); and (*c*) those corrinoids which undergo photoreduction to yield a cobaltous complex. Corrinoids which undergo photoreduction include the alkyl derivatives (126, 127, 144, 160, 161), 5′-deoxyadenosyl-B_{12} and its analogues (126, 148, 149, 162, 163), and the corrinoid-sulphonates (153, 155). Photochemical reactions of the corrinoid thiol complexes have been observed, but are complicated by secondary irreversible reactions (9, 155, 160).

Photolysis of 5′-deoxyadenosyl-B_{12} in the presence of oxygen results in the formation of OH-B_{12}, adenosine-5′-aldehyde and 8,5′-cyclic-adenosine; in the absence of oxygen, however, B_{12r} and 8,5′-cyclic-adenosine are the major products (117). In contrast to these results, the anaerobic photolysis of 5′-deoxy-2′,3′-isopropylideneuridinyl-B_{12} yields OH-B_{12} and 2′,3′-isopropylidenecyclodihydrouridine (164). The photodecomposition of methyl-B_{12} has been studied in aqueous solution at different oxygen concentrations (31, 120, 161). In the presence of an excess of oxygen, methyl-B_{12} is photolyzed yielding OH-B_{12} and formaldehyde as the main products (120, 161). In the absence of oxygen, methyl-B_{12} or ethyl-B_{12} are stable in light (9, 31), but in the presence of limiting oxygen concentrations B_{12r} and a mixture of olefins and paraffins were obtained (31). Hogenkamp (161) has studied the fate of the methyl radical from [^{14}C]-methyl-B_{12} as a function of oxygen concentration. Photolysis in the presence of only a trace of oxygen is slow, and yields B_{12r}, formaldehyde, methane, and ethane. He suggested that both paraffins are probably formed via hydrogen or methyl abstraction from the corrin ring by a methyl radical. However, the reaction product, B_{12r}, was not fully analyzed and more work is needed to clarify its nature. In the presence of thiols, the alkyl group liberated on photolysis from alkyl-B_{12} or 5′-deoxyadenosyl-B_{12} is converted into S-alkyl derivatives (148). Anaerobic photolysis of carboxymethyl-B_{12} or 2-ethoxycarbonylethyl-B_{12} yields acetic acid or ethyl acrylate, respectively (127). Photodecomposition of 2,3-dihydroxypropyl-B_{12} under anaerobic or aerobic conditions yields glycerol or glyceraldehyde and glyceric acid as well (145).

These observations indicate that the photolytic cleavage of the alkylcorrinoids involves the homolytic fission of the cobalt-carbon bond. Therefore, it is evident that all these alkylcorrinoids are cobaltic complexes. The comparison of the first order photolysis rates (33, 160) with the inductive effects of the alkyl groups does not show any correlation (33). It seems likely that the rate of B_{12r} oxidation depends on secondary reactions of the alkyl peroxide radical (160).

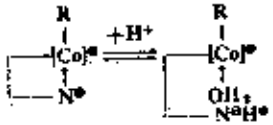
Reaction with cyanide in the dark.—Alkaline cyanide converts 5′-deoxyadenosyl-B_{12} into dicyano-B_{12} by splitting off adenine and the cyanohydrin of erythro-3,4-dihydroxy-1-penten-5-al (167). Other corrinoids with a 5′-deoxynucleoside ligand react in the same way (126, 148, 149, 168). These reactions have been proposed to involve a nucleophilic displacement of the nucleoside ligand (167) and it is probably a concerted cleavage which does not require a carbanion leaving group (6). The simple alkylcorrinoids are stable to alkaline cyanide (31, 33, 126), presumably because the alkylcarbanions are poor leaving groups. However, alkylcorrinoids are readily converted to dicyanocorrinoids if the alkyl group contains a strong electrophile and is therefore able to leave as a carbanion (33).

All the corrinoids containing a Co-S bond react rapidly with alkaline cyanide to give the dicyanocorrinoids (153, 155).

Reaction with acids in the dark.—5′-Deoxyadenosyl-B_{12} is converted to OH-B_{12}, adenine and erythro-2,3-dihydroxy-1-penten-5-al on mild acid hydrolysis (169). Its nucleoside analogues, 2′,5′-dideoxyadenosyl-B_{12} and 5′-deoxythymidyl-B_{12}, yield under these conditions OH-B_{12}, adenine or thymine, and *trans*-2,4-pentadiene-4-al (148). It is apparent that the rate of acid hydrolysis of the Co-C bond is determined by the stability of the nucleoside glycosyl linkage toward acid hydrolysis. This mechanism of decomposition differs from that with cyanide because the heterolytic cleavage of the Co-C bond is initiated by protonation of the ring oxygen (148). The acid hydrolysis of 2-hydroxyethyl-B_{12} or 2-methoxyethyl-B_{12} (33), and of 2-aminoethyl-B_{12} (170), in which the protonated alkyl groups are strong electrophiles, agrees with this mechanism. Alkylcorrinoids not acquiring a positive charge in the alkyl ligand are not decomposed under acid conditions or are decomposed only slowly (31, 33, 126, 144).

The alkyl-B_{12} derivatives are protonated by cold dilute mineral acid

which produces a shift from red to yellow (153, 155). In contrast, OH-B_{12}, CN-B_{12}, SCN-B_{12} and HSO_4-B_{12} require concentrated mineral acid to bring about a similar color change (122, 155). These studies indicated a correlation between the inductive effect of the alkyl substituent and the strength of the Co-benzimidazole coordinative linkage. This is illustrated, for example, by the structures:



Where R = CH_2-CH_3, the pK_a values for the indicated transition is 3.87; whereas when R = CH_2-CH_2-CN, the pK_a value is 2.95. Two explanations for the color change in the alkyl-B_{12} series have been advanced. One interpretation suggests that it results only from the cleavage of the Co-benzimidazole coordinative linkage (171). An extension of this interpretation (122) suggests that in acid solution a protonation has occurred in the chromophore, likely at position C-10 (31, 122, 136). However, this latter interpretation seems unlikely, because the 10-halogen-alkyl-B_{12} derivatives show the same large hypsochromic shift on acidification as the alkyl-B_{12} derivatives (106, 120).

Reaction with iodine.—5'-Deoxyadenosyl-B_{12} is split by iodine yielding iodo-B_{12} and 5'-iodo-5'-deoxyadenosine (172). The rate of decomposition of alkylcorrinoids by iodine is greatly influenced by the alkyl ligand. The following order of lability has been reported (172): 5'-deoxyadenosyl-B_{12} ≈ 5'-deoxyadenosylcobinamide ≫ ethyl-B_{12} > methyl-B_{12}. Experiments with iodine monochloride suggest that the cleavage is caused by an iodine cation.

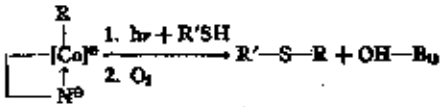
Alkaline oxydation.—In CN-B_{12} an oxidative substitution reaction occurs at position C-8 of ring B with remarkable ease, indicating that this center is easily converted into a trigonal intermediate. Air oxidation of CN-B_{12} in alkaline solution causes cyclization of the acetamide side chain in ring B to a lactam, which has been called dehydro-vitamin B_{12} (173). No oxidative cyclization takes place with 5'-deoxyadenosyl-B_{12}, methyl-B_{12} or sulphito-B_{12} (120, 174).

Substitution reactions.—A second oxidative cyclization of the acetamide side chain in ring B of CN-B_{12} has been described (173). Action of chloramine T or bromine water at pH 4 at first causes cyclization to lactone-B_{12} and further reaction of an excess of these agents causes substitution of chlorine or bromine into the chromophore yielding the halogen-lactone-B_{12} (173). The reaction of 5'-deoxyadenosyl-B_{12}, methyl-B_{12}, or sulphito-B_{12} with chloramine T in 0.5 M acetic acid takes a different course (106, 120, 136, 174). In these products the first equivalent of chloramine T results in substitution of chlorine into the chromophore without cyclization; an excess of chloramine T leads to the formation of the corresponding monochlorolactones. Substitution of the chromophore of CN-B_{12} before cyclization occurs is possible in

glacial acetic acid. Thus N-bromosuccinimide yields 10-bromo-CN-B_{12} and cyanolactone-B_{12} (106). NOCl or NO_2Cl gives 10-nitro-CN-B_{12}, which is easily reduced to 10-amino-CN-B_{12} (106). In contrast to these results, the reaction of chloramine T or N-bromosuccinimide with cyanocobinamide in 0.5 M acetic acid or glacial acetic acid yields only 10-halogeno-cyanocobinamide, without lactonization (175).

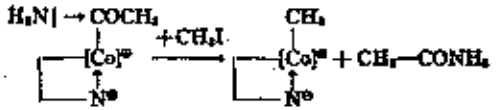
These results show such a substitution in the chromophore of corrinoids with electrophilic agents in the alkyl form, as well as in the cyano form. A consideration of the electronic absorption spectra from chloro-, bromo-, nitro-, amino-, and acetamino-B_{12} leads to the conclusion that the most reasonable position for these substituents is C-10 of the corrinoids (See Fig. 1, R = Cl, Br, NO_2, NH_2, or CH_2-CONH) and agrees with the proton magnetic resonance spectrum of 10-chloro-CN-B_{12} (34).

Transfer reactions.—Anaerobic photolysis of alkyl-B_{12} derivatives yields an intermediate alkyl radical which in the presence of thiols is transferred to these (165):

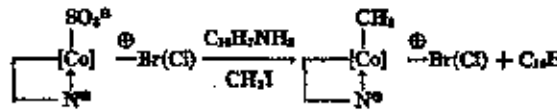


It has been shown, also, that photolysis of methyl-B_{12} in presence of 1,4-naphthoquinone yields 2-methyl-1,4-naphthoquinone (31).

Another transfer reaction has been found with acyl-B_{12} derivatives (105). When acetyl-B_{12} reacts anaerobically with a nucleophilic agent, such as ammonia, in the dark and in the presence of methyl iodide, methyl-B_{12} and acetamide are obtained. From this fact an intermediary formation of B_{12} can be inferred.



10-Chloro- and 10-bromo-sulphito-B_{12} yield, by the electronic direction of the halogen substituents with β-naphthylamine as nucleophilic agent in the presence of air and in the dark, the corresponding aquo derivatives and β-naphthylsulphamic acid. If the transfer reactions are carried out anaerobically in the dark and in the presence of methyl iodide, β-naphthylsulphamic acid and the 10-chloro- or 10-bromo-methyl-B_{12} (9, 106) are formed.



Structure of the corrinoids.—At first it was not clear from the X-ray analysis of 5'-deoxyadenosyl-B_{12} whether the corrinoid ligand system had

the usual six double bonds as in the case of the cyanocorrinoids (1). Reduced forms in the alkylcorrinoids with a tetrahedral C-19 position (176) or with a hydrogenated 9-22 bond (174) have been suggested, but the latter view has been modified (120). The results of the following experiments indicate that the corrinoid ligand system of the 5'-deoxyadenosyl-B_{12} and the alkylcorrinoids is the same as in the cyanocorrinoids and both contain trivalent cobalt. (*a*) The synthesis of methyl-B_{12} from OH-B_{12} in water-containing tritium leads to a radioactive product with tritium in the methyl group bound to the cobalt, but no tritium incorporation into the corrinoid ligand system was detected (120). (*b*) The controlled electroreduction of OH-B_{12} to B_{12a} requires two electrons (104, 137). B_{12a} prepared in this way reacts with methyl iodide yielding methyl-B_{12} and no additional electrons are available to reduce the corrinoid ligand system (137). (*c*) The magnetic susceptibilities of cyano-, or dicyano-B_{12} and 5'-deoxyadenosyl-B_{12} in both red and yellow forms are diamagnetic (32). Therefore the alkylcorrinoids can be regarded as diamagnetic complexes of trivalent cobalt with a carbanion as ligand.

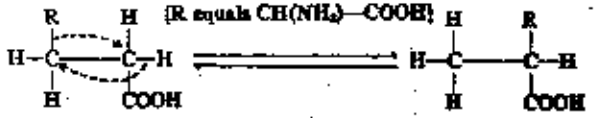
Enzymatic Reactions

In 1958 Barker et al. (21) isolated a coenzyme form of α-(adenyl) cobamide and demonstrated its participation in the isomerization of glutamate to β-methylaspartate, catalyzed by an enzyme present in *Cl. tetanomorphum*. Meanwhile, the knowledge of the enzymatic functions of corrinoids has rapidly increased. However, in all corrinoid-dependent enzymatic reactions the mode of action of these coenzymes is unknown or only partly elucidated.

Reactions Involving 5'-Deoxyadenosylcobamides

Glutamate mutase.—The first step in the fermentation of L-glutamate by extracts of *Cl. tetanomorphum* is its conversion to L-*threo*-β-methylaspartate by the 5'-deoxyadenosylcobamide-dependent glutamate mutase system (177, 178). Evidence was obtained for the existence in this system of two separable proteins, called the E and S components (178). Component E has now been purified and completely freed of S component activity and of β-methylaspartase (179). Both purified component E with a molecular weight of approximately 128,000 and partially purified component S are essential for glutamate mutase activity under either aerobic or anaerobic conditions. The binding of 5'-deoxyadenosyl-B_{12} to component E is relatively weak (179).

This enzymatic reaction can be thought to involve a reversible transfer of a glycine residue and of a hydrogen by way of a double rearrangement (177, 180).



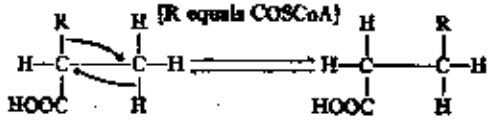
The possibility that free ammonium ion, α-iminoglutarate, glycine, acrylic acid or a free proton are intermediates was eliminated by tracer experiments (177, 179). The intramolecular hydrogen transfer occurs in a stereospecific way with inversion of the configuration of C-4 of glutamate (180). The 5'-deoxyadenosyl group is absolutely necessary for coenzyme activity in the glutamate mutase reaction. Its replacement by other ligands, such as 5'-deoxyuridine, 5'-deoxythymidyl, 2',5'-dideoxyadenosyl, or alkyl groups, leads to loss of activity or to competitive antagonism (148, 177). Other corrinoid derivatives, such as 5'-deoxyadenosyl-10-chloro-B_{12}, are also inactive (181).

L-Methylmalonyl-CoA mutase.—This reaction, demonstrated in both microorganisms and animal tissues, is important in the metabolism of propionate (182, 183). It involves the carboxylation of propionyl-CoA to D-methylmalonyl-CoA, isomerization of the latter to the L-enantiomorph and then its conversion to succinyl-CoA (184–188). L-Methylmalonyl-CoA mutase has now been obtained in purified form from *P. shermanii* (189) and from sheep liver (190). The characterization of this enzyme from both sources showed some differences.

The purified holoenzyme from sheep liver has a molecular weight of 165,000 and contains roughly 2 moles of 5'-deoxyadenosyl-B_{12} per mole of enzyme. While the holoenzyme is relatively stable, the apoenzyme, which has a similar molecular weight, is very unstable and sensitive to sulfhydryl binding reagents. The full activity of the apoenzyme can be restored by the addition of 5'-deoxyadenosyl-B_{12}. It was assumed that holoenzyme formation involves at least a two-point attachment between the coenzyme and apoenzyme and that sulfhydryl groups are one binding site of the protein (190).

A molecular weight of 56,000 was obtained for the bacterial enzyme (189). The activity of this enzyme does not depend on sulfhydryl groups and the coenzyme could readily be separated (189). The reasons for the differences between the mammalian and bacterial enzymes were discussed by Ochoa & co-workers (190).

The isomerization of methylmalonyl-CoA, in analogy to that of glutamate, occurs by an intramolecular shift of the carbonyl thioester group from the α- to the β-carbon of the propionic acid residue with a simultaneous hydrogen shift (191–193). Again, no solvent hydrogen is incorporated during the isomerization reaction (194).



Several hypothetical mechanisms have been proposed (195–197).

Changes in the 5'-deoxyadenosyl group have a pronounced effect on coenzymatic activity, whereas modifications of the bases in the nucleotide part are relatively unimportant (182, 183). 5'-Deoxyadenosylcobinamide

has no activity (189). Overath et al. (188) have found a low activity of this compound, certainly caused by the presence of 5′-deoxyadenosyl-B_{12}.

The presence of the methylmalonyl-CoA system also has been demonstrated in cell-free extracts from rat liver (198), ox liver (199), and sheep kidney (200). The coenzyme also is partly utilized in this way by *Ochromonas malhamensis* (201). One function of vitamin B_{12} in man is to act as a source of coenzyme for the methylmalonyl-CoA-mutase (202).

Dioldehydrase.—This enzyme catalyzes the conversion of 1,2-propanediol to propionaldehyde or of 1,2-ethanediol to acetaldehyde. Dioldehydrase activity was found in cell-free extracts of *A. aerogenes* (203–205) and has been purified 200-fold (206). A similar conversion of glycerol to β-hydroxypropionaldehyde has been demonstrated with extracts from *Lactobacillus* 208-A (207, 208) and from *A. aerogenes* PZH 572 (209). All these reactions depend on 5′-deoxyadenosylcobamides.

Studies to elucidate the mechanism of the dioldehydrase reaction have shown that this reaction involves an intramolecular 1,2-hydrogen shift (210). The enzymatic conversion of stereospecifically labelled 1,2-propanediol takes the following course (211):

$$\text{D-CH}_3\text{CHOHCHDOH} \rightarrow \text{CH}_3\text{CHDCHO}$$
$$\text{L-CH}_3\text{CHOHCHDOH} \rightarrow \text{CH}_3\text{CH}_2\text{CDO}$$

The steric course of these reactions was explained by assuming an interaction of the enzyme and substrate that involves the binding of the two hydroxyl groups at a specific site of the protein. The dioldehydrase in the presence of 5′-deoxyadenosyl-B_{12} can act both as an electron donor and as an electron acceptor (212). These transformations and the ability of the enzyme to bring about a heterolytic cleavage of the cobalt-carbon bond in 5′-deoxyadenosyl-B_{12} (206) are remarkable features of the mechanism of cobamide coenzyme-dependent reactions.

Numerous nucleoside analogues of 5′-deoxyadenosyl-B_{12} and alkylcorrinoids have been tested in the dioldehydrase system. Most of these derivatives, including CN-B_{12} and OH-B_{12} were either inactive or competitive inhibitors (141, 144, 147, 148, 149, 209). Only 2′,5′-dideoxyadenosyl-B_{12} (148), 5′-deoxy-N_6-methyladenosyl-B_{12} (149) and 5′-deoxyadenosylcobinamide (209) showed activity, but all were less active than 5′-deoxyadenosyl-B_{12}.

Ethanolamine deaminase.—An inducible ethanolamine deaminase from an ethanolamine-fermenting *Clostridium* requires 5′-deoxyadenosyl-B_{12} or α-(benzimidazolyl)-5′-deoxyadenosylcobamide (213). This enzyme converts ethanolamine to acetaldehyde and ammonia.

$$\text{H}_2\text{N}^{\oplus}\text{—CH}_2\text{—CH}_2\text{—OH} \rightarrow \text{NH}_4^{\oplus} + \text{CH}_3\text{—CHO}$$

This reaction is very similar to the transformation of 1,2-diols catalyzed by dioldehydrase. The deaminase is also strongly inhibited by OH-B_{12}, CN-B_{12} and methyl-B_{12}, but cell-free extracts are able to synthesize 5′-deoxyadenosyl-B_{12} from these compounds. An analogous reaction seems to occur with choline.

Ribonucleotide reductase.—A relationship between vitamin B_{12} and the synthesis of deoxynucleosides from ribonucleosides, by direct reduction, was established by numerous studies (see reviews 3, 9, 13, 14). Blakley & Barker (214) have recently obtained evidence of a 5′-deoxyadenosyl-B_{12} requirement for the reduction of cytidine monophosphate to deoxycytidine monophosphate by crude extracts of *Lactobacillus leichmanii*. Tracer experiments have indicated that the glycosidic bond in the ribonucleotides is not broken during the enzymatic reduction (108, 214, 216). The results obtained with fractionated extracts suggested that the substrates for the reductase are ribonucleoside triphosphates and that the purified reductase shows much greater activity with guanosine triphosphate than with any other tested ribonucleotide (217). This enzyme requires dihydrolipoic acid in addition to 5′-deoxyadenosyl-B_{12}. The ribonucleotide reductase of *L. leichmanii* is repressible (108).

Maximal reductase activity of this system occurs with 5′-deoxyadenosyl-B_{12} and α-(benzimidazolyl)-5′-deoxyadenosylcobamide (108, 214, 215, 219). The former is probably the major naturally occurring corrinoid in *L. leichmanii* (108, 218). Most other corrinoids tested were virtually inactive, but 2′,5′-dideoxyadenosyl-B_{12} is active (215). The parallelism with the activity of corrinoids in the dioldehydrase from *A. aerogenes* is remarkable (148). This points to a similar mechanism of reaction for both enzymatic systems.

Lysine degradation.—The conversion of lysine to butyrate, acetate and ammonia by cell-free extracts of *Cl. sticklandi* and *Clostridium* M-E, is 5′-deoxyadenosylcobamide-dependent (220, 221). This oxidation-reduction process is coupled with formation of ATP from ADP and orthophosphate. The specific step in which the cobamide coenzymes participate has not been identified.

Reactions With Other Corrinoids

Methionine synthesis.—Two different pathways for methionine methyl formation have been reported (see 222, 223). One shows a requirement for vitamin B_{12} and the other does not. The vitamin B_{12}-independent enzyme system, which occurs in *E. coli* PA-15 (224), *E. coli* W (225), *A. aerogenes* (226), and in extracts of spinach (223), requires homocysteine, 5′-methyltetrahydropteroyltriglutamate, and Mg^{++}.

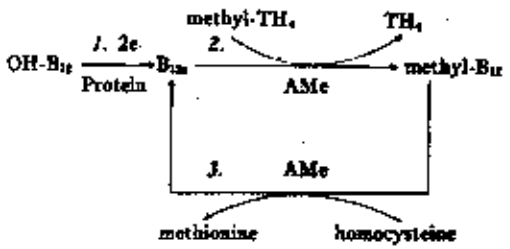
The vitamin B_{12}-dependent pathway requires as additional cofactors reduced flavin adenine dinucleotide and S-adenosylmethionine (AMe), together with 5-methyltetrahydropteroyl mono- or triglutamate (methyl-TH_4) as methyl donor (see 222, 223). This transmethylase system was found in the *E. coli* strains mentioned above, in *A. aerogenes* (227), and in mammalian liver (228, 229).

The B_{12}-dependent methyltransferase reaction proceeds according to the equation:

5-Methyltetrahydrofolate + homocysteine

$$\xrightarrow[\text{reducing system}]{\text{AMe, cobamide protein}} \text{methionine} + \text{tetrahydrofolate}$$

5-Methyltetrahydrofolate-homocysteine transmethylase, which catalyzes this reaction, contains a vitamin B_{12} derivative as a prosthetic group (222, 223). *E. coli* PA-15 forms a stable holoenzyme when grown in the presence of CN-B_{12}, but forms only the apoenzyme in the absence of the vitamin (230). *A. aerogenes* produces sufficient B_{12} to form the holoenzyme without addition of the vitamin (227). Methyl-B_{12} can serve as an intermediate in the transferase reaction (231, 232), and this has focused attention on the possible role of methyl-B_{12} as a prosthetic group in the holoenzyme. Studies on the formation of the holoenzyme *in vitro* have shown (222, 223) that a stable holoenzyme is formed with OH-B_{12} or CN-B_{12} only in the presence of the reducing system. However, with methyl-B_{12} as cofactor, no reduction is necessary for holoenzyme formation. Since B_{12a} also requires a reducing system to form the holoenzyme, a two-electron reduction of the vitamin appears to take place (233). Based on these considerations the biosynthesis of methionine by the B_{12}-dependent system (222, 223, 232–236) seems to follow the accompanying cyclic pathway.



In the Reactions 2 and 3 the methyl group will probably be transferred as a methylcarbonium ion. However, the catalytical role of S-adenosylmethionine, required in Reactions 2 and 3, is not known (222, 223, 236, 237). Possibly it functions as an allosteric regulator (8, 236). The transmethylase from hog liver and *E. coli* catalyzes the transfer of the methyl group from methyltetrahydrofolate to cobinamide (223, 238). Another vitamin B_{12} derivative, not identical to 5'-deoxyadenosyl-, methyl-, or OH-B_{12}, has been isolated from *Bacillus megaterium* (239). It stimulates the apoenzyme of the transmethylase of *E. coli*.

Methane formation.—Cell-free extracts of *Methanosarcina barkeri* catalyze the reduction of [^{14}C]-methyl-B_{12} to $^{14}CH_4$ (240, 241) and also the formation of methane from methanol, formaldehyde, or carbon dioxide (240, 241). The amount of $^{14}CH_4$ produced from $^{14}CH_3OH$ is decreased by

addition of unlabelled methyl-B_{12}; however, $^{14}CH_4$ formation from [^{14}C]-methyl-B_{12} is not influenced by unlabelled methanol (241). The demonstration of the enzymatic formation of methyl-B_{12} from methanol and B_{12a} in extracts of *M. barkeri* is further evidence for a cobamide-dependent reduction of methanol (241). The formation of methane and B_{12a} from methyl-B_{12} was shown in extracts of *Methanobacterium omelianskii* (242, 243). This ability seems to be a common property of the methane bacteria (246). It was postulated that B_{12a} is an intermediate both in the conversion of methyl-B_{12} to methane (243) and in the enzymatic synthesis of methyl-B_{12} from methanol (241). However, from the reactivity of B_{12a} discussed above (see pp. 414 and 416), this compound is very probably only a transient intermediate in both enzymatic reactions.

In extracts of *M. omelianskii* 5-methyltetrahydrofolate seems to be an intermediate in the formation of both methane and methionine from carbon dioxide, pyruvate, or serine (245). Methyl-B_{12} may not be an obligatory intermediate in this case (245), in agreement with the finding that no methylcorrinoid could be detected in *M. omelianskii* (73).

Acetate formation.—Cell-free extracts from *Cl. thermoaceticum* catalyze the incorporation of the methyl group of methyl-B_{12} selectively into the methyl group of acetate (246). This enzymatic reaction requires carbon dioxide and pyruvate. The same extracts also catalyze the synthesis of [^{14}C]-methyl-B_{12} from $^{14}CO_2$ and reduced cobalamin (247). Three fractions of the extract are required for the synthesis of acetate from methyl-B_{12}, but one of these fractions can be replaced by ferredoxin (247). However, it seems likely that none of the natural intermediates in acetate synthesis is a cobalamin derivative (248). Surprisingly, the methylated products isolated α-(5-methoxybenzimidazolyl)-methyl-cobamide and methylcobyric acid (65). Both corrinoids labelled in the methyl group have been isolated from intact cells of *Cl. thermoaceticum* grown in glucose medium and exposed to $^{14}CO_2$ for a short time (66). Cell-free extracts from this microorganism catalyze the selective conversion of the labelled methyl group of these methylcorrinoids to the methyl group of acetate in the presence of pyruvate. Apparently methylcobyric acid is the better precursor of the two; probably a carboxymethylcorrinoid is a natural intermediate. Further evidence for the latter compound is the fact that extracts of *Cl. thermoaceticum* produce acetate from carboxymethyl-B_{12} in the presence of reduced nicotinamide adenine dinucleotide phosphate (66).

Concluding Remarks

The growing understanding of corrinoid chemistry will be the basis for a deeper knowledge of the biochemistry of these fascinating and unique substances of low molecular weight. It seems of the greatest importance, therefore, to show what influence changes in the corrinoid ligand system and in the axial ligands have on the reactivity of the molecule. Because they throw light on these problems, study of corrinoids of modified structure also leads

to a better understanding of the biochemistry of B_{12}. Investigations on the biosynthesis of the B_{12} molecule must show not only how it is formed, but also why it must have just this structure to perform its numerous biological functions.

At least nine different cell-free enzymatic reactions that are dependant on 5'-deoxyadenosyl-, methyl-, or carboxymethylcorrinoids are now known. In some of these reactions, a reduced corrinoid with Co^{2+} very probably functions as the active prosthetic group. Above all, the intramolecular double rearrangement, dependent on 5'-deoxyadenosylcobamide, is still mysterious. The importance to mammalian biochemistry of an understanding of vitamin B_{12} is increasing, since at least two of the enzymatic reactions dependent on corrinoid coenzymes occur in mammals including man.

The ultimate goal would be the isolation in crystallized form of a corrinoid-containing enzyme whose structural elucidation in three dimensions would make possible a thorough understanding of the intriguing set of reactions for which these coenzymes are required.

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Generalized Malabsorption, Failure to Thrive

Result of Cyanocobalamin Deficiency

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Failure to thrive, generalized malabsorption, and pernicious anemia caused by cyanocobalamin deficiency occurred in a child. There was a beneficial hematologic response and improvement in the malabsorption phenomena following parenteral administered cyanocobalamin. However, a selective defect in cyanocobalamin absorption persisted. Studies related to intrinsic factor activity included abnormal results of Schilling tests which were not corrected by the administration of exogenous intrinsic factor or small intestinal juice. However, intrinsic factor activity was present in the patient's gastric juice as demonstrated by in vitro and in vivo assays. These findings suggest that patients with the Imerslund-Gräsbeck syndrome have a cyanocobalamin deficiency caused by a specific defect in the small intestinal mucosa metabolism of the intrinsic factor-cyanocobalamin complex. Cyanocobalamin deficiency may occasionally be associated with generalized malabsorption.

Megaloblastic anemia caused by cyanocobalamin deficiency is relatively uncommon in the pediatric age group. One form of pernicious anemia is associated with a congenital absence of gastric intrinsic factor activity with no other abnormalities.[1] The findings in patients with juvenile pernicious anemia are similar to those in adults with classical addisonian pernicious anemia. Achlorhydria, absent intrinsic factor activity and antibodies to parietal cells, and intrinsic factor may be present.[2] Polyendocrinopathy may also occur in this group. Megaloblastic anemia due to cyanocobalamin deficiency, despite the presence of intrinsic factor activity, has been described by Imerslund[3] and Gräsbeck et al.[4] A familial occurrence and benign proteinuria may be present in this syndrome. Since generalized intestinal malabsorption as a complication of Imerslund-Gräsbeck syndrome has not been previously described, the following case is presented.

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Report of a Case

The patient was a 16-month-old white boy with a six-month history of increasing pallor and failure to thrive. During this time frequent, foul smelling stools were noted associated with a 1.5 kg (3 lb 2 oz) weight loss.

There was no family history of consanguinity. The patient's brother died at 2 years of age with severe anemia; his hemoglobin level was 2 gm/100 ml. Autopsy findings included a megaloblastic maturation defect of the bone marrow.

Pallor was noted during the physical examination. The child was poorly developed with muscular wasting, apparent in the gluteal region. No hepatosplenomegaly was present. The results of the neurological examination were normal. No ataxia was present and the deep tendon reflexes were active.

Pancytopenia was present (Table 1). The hemoglobin level was 5.5 gm/100 ml; macrocytosis was found on examination of the peripheral smear; the mean corpuscular volume was 114 microcuries, and the reticulocyte count 2.7%. Bone marrow examination revealed a reversed myeloid-erythroid ratio of 1:6. Numerous megaloblasts were present with nuclear-cytoplasmic dissociation. Gastrointestinal studies showed generalized malabsorption (Table 2). Stool examination results for ova and parasites were negative.

Qualitative urinalysis revealed proteinuria (1+); maximum urinary protein excretion for 24 hours was 240 mg. Urine cultures were negative. An intravenous pyelogram was negative. Creatinine clearance and urine amino acid chromatography were normal.

ıd Megaloblastic Anemia

The serum cyanocobalamin level was low, 20μμg/ml (normal, 150μμg to 900μμg/ml). The urinary methylmalonic acid level was 236 mg/24 hr (normal, < 20 mg); serum iron level was 220μg/100 ml with 93% saturation of the iron binding capacity. Serum folate level was 12 ng/ml (normal, 2 to 8 ng/ml). There was no hematologic response following consecutive courses, of seven days each, of 100μg of orally administered folic acid, 100μg of intramuscularly administered folic acid, and 100μg of intramuscularly administered folinic acid. A Schilling test showed less than 2% excretion of cyanocobalamin labeled with cobalt 60. Despite the addition of 50 mg of hog intrinsic factor of known potency, a repeat Schilling test again showed less than 2% excretion. Five days after the parenteral administration of 1,000μg of cyanocobalamin the reticulocyte count rose from 2.7% to 25%. Because of the defect in cyanocobalamin absorption, treatment with 1,000μg of cyanocobalamin intramuscularly administered once a month was begun. After four months of therapy there was marked improvement in the hematologic findings and partial correction of the malabsorption phenomena (Tables 1 and 2). However, a repeat Schilling test was abnormal with less than 2% excretion. A filtered specimen of the patient's gastric juice was administered to an adult who had classical pernicious anemia with absent intrinsic factor activity. His abnormal Schilling test (< 2% excretion) was cross-corrected to 12% excretion.

When evaluated after 17 months of cyanocobalamin therapy the peripheral blood findings were normal (Table 1). The Schilling test was again abnormal with less than 2% excretion. Several modified Schilling tests with the addition of intrinsic factor and small intestinal juice (ph > 10.0) from two sources, an ileostomic patient and a normal volunteer, also demonstrated the patient's defect in cyanocobalamin absorption with less than 2% excretion in all the tests.

During the 17 months of therapy with cyanocobalamin, there was a change in the weight grid channels from less than the third percentile to the fiftieth percentile. Minimal proteinuria, however, persisted.

Peripheral blood cell counts on the patient's parents and a female sibling showed normal levels. At 1 year of age the sister's serum cyanocobalamin level was 260μμg/ml, and a bone marrow aspiration showed a normal maturation pattern.

Table 1.—Hematologic Findings

	Present at Time of Diagnosis	Following Cyanocobalamin Therapy	
		4 mo	17 mo
Hemoglobin (gm/100 ml)	5.5	10.9	13.0
White blood cell count/cu mm	4,000	6,120	6,900
Platelet count/cu mm	30,000	176,000	265,000
Bone marrow	Megaloblastic maturation	Normal maturation	

Table 2.—Gastrointestinal Studies

	At the Time of Diagnosis	After 4 mo of Cyanocobalamin Therapy*
Patient's weight	8.4 kg (< third percentile)	11.2 kg (tenth percentile)
Dietary fat absorption (normal 90%, intake)	75%	92%
Gastric acidity	Present	
Glucose tolerance test	Flat curve	Normal
Lactose tolerance test	Flat curve	
Xylose excretion	3.5%	9.4%
Gastric parietal cell antibodies	None present	
Small bowel roentgenograms	No abnormalities	
Small bowel biopsy	Mild blunting of villi with plasma cell infiltration of the lamina propria	

* Cyanocobalamin, 1,000 μg, administered intramuscularly once per month.

Comment

A 16-month-old boy with the Imerslund-Gräsbeck syndrome and generalized malabsorption was treated. Pernicious anemia developed despite the presence of intrinsic factor activity in the patient's gastric juice. In addition, familial occurrence and benign proteinuria were present. The familial aspect of the disease was manifested by the autopsy finding of megaloblastosis in a male sibling. The patient's renal function studies revealed the persistent finding of minimal proteinuria with no other demonstrable abnormalities.

The low serum cyanocobalamin level was caused by a selective defect in cyanocobalamin absorption. The inability to correct the patient's abnormal Schilling test with exogenous intrinsic factor of known potency indicated that intrinsic factor deficiency was not the cause of this defect in absorption. Intrinsic factor activity was present in his gastric juice, as determined by in vitro[7] and in vivo evaluation. The in vivo assay consisted of a cross-correction of an abnormal Schilling test in an adult with classical pernicious anemia and absent intrinsic factor activity by the administration of our patient's gastric juice. These findings indicate that the selective defect in cyanocobalamin absorption was not caused by the absence of intrinsic factor activity.

Herbert et al[6] postulated the presence of releasing factor activity in the small intestine which was required for the cleavage of the cyanocobalamin-intrinsic factor complex which resulted in making cyanocobalamin available for absorption. Colle et al[7] reported the correction of an abnormal Schilling test in a patient with the Imerslund-Gräsbeck syndrome following the administration of small intestinal juice. This supported the concept that a specific intestinal factor was required for absorption of the cyanocobalamin-intrinsic factor complex. However, Ben-Bassat et al[8] were unsuccessful in correcting the malabsorption of cyanocobalamin following the administration of normal intestinal juice in 11 of 13 patients with a specific defect in absorption of cyanocobalamin. We also could not correct our patient's abnormal Schilling test with the administration of small intestinal juice from two sources.

The cyanocobalamin deficiency may have been related to an abnormality in the metabolism of the intrinsic factor-cyanocobalamin complex by the small intestine mucosal cells. Donaldson et al[9] showed that the presence of intrinsic factor increased the uptake of cyanocobalamin by the brush borders and microvillous membranes obtained from the distal half of intestinal cells in hamsters. Cooper[10] has shown that the formation of an intrinsic factor-cyanocobalamin complex is required for absorption of cyanocobalamin. This complex is transported to a receptor site in the small intestine where hydrolysis results in the release of cyanocobalamin for absorption. The data presented related to intrinsic factor activity suggest that patients with Imerslund-Gräsbeck pernicious anemia have a defect in the small bowel mucosal cell metabolism of the intrinsic factor-cyanocobalamin complex that results in the malabsorption of cyanocobalamin.

The causal relationship of cyanocobalamin deficiency and generalized malabsorption was established by the improvement in the clinical and hematologic findings and the partial correction of the laboratory findings of malabsorption following the parenteral administration of cyanocobalamin. While on therapy, significant weight gain occurred. The oral glucose tolerance test results were normalized, and fat and xylose absorption was improved. Although morphologic changes such as shortening of villi and megaloblastosis have been described in mucosal cells from small intestinal biopsies in patients with pernicious anemia, the physiologic significance of these findings is not clear.[11] The report of generalized malabsorption in adults with pernicious anemia[12] and the present findings suggest that these changes may occasionally have clinical significance.

Nonproprietary and Trade Names of Drug

Cyanocobalamin—*B-[illegible] Co, Lemon 12, Cotel, Ducibin, Dodecavite, Norvitin.*

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Preliminary Communication

SEMINAL VITAMIN B_{12} AND STERILITY

IN assessing male fertility the routine laboratory investigations on semen are, as a general rule, limited to measurements of the volume of the ejaculate and the sperm density, and estimations of the proportion of motile forms and the proportion of spermatozoa having normal morphological characteristics.

Various workers have reported that, when the proportion of morphologically normal spermatozoa is low, there is often an associated decrease in the proportion of motile forms and a lowering of the sperm-count, suggesting that the changes might be a consequence of a maturation defect of the spermatozoa.

Busch [1] showed that when stored bull semen was used in artificial insemination the addition of vitamin B_{12} improved its eventual motility and enhanced its degree of fertility. It was therefore decided to investigate vitamin-B_{12} levels in serum and in seminal fluid in order to explore any possible role for this vitamin in human sperm maturation.

THE STUDY

The study concerns the seminal fluid and serum of thirty consecutive patients who attended a sterility clinic. Sperm-counts, estimations of sperm motility, and assessments of the proportion of morphologically normal forms were made. A specimen was regarded as morphologically within normal limits if less than 30% of the sperms showed atypical structural features. Estimations of the vitamin-B_{12} levels in the seminal fluid and the serum were carried out by the *Euglena gracilis* method.[2,3] The ratio of vitamin B_{12} in the semen to vitamin B_{12} in the serum was designated the semen/serum quotient.

RESULTS

In eighteen of the thirty patients the specimens were regarded as normal. The vitamin-B_{12} levels in their semen and in the semen of two known fertile donors were found to be higher than the levels in the corresponding serum specimens. In these cases therefore the semen/serum quotient (as designated above) was *greater* than 1.

In twelve cases the specimens were regarded as abnormal (i.e., more than 30% of the spermatozoa showed morphological abnormalities). The vitamin-B_{12} levels in the semen and serum in these cases showed an inverse ratio, the quotient being *below* 1, with the exception of one case. In other words, in these cases the seminal B_{12} level was less than the serum level. The findings are summarised in the figure.

Three of these cases had azoospermic semen, and in each of these the vitamin-B_{12} factor was less than 1.

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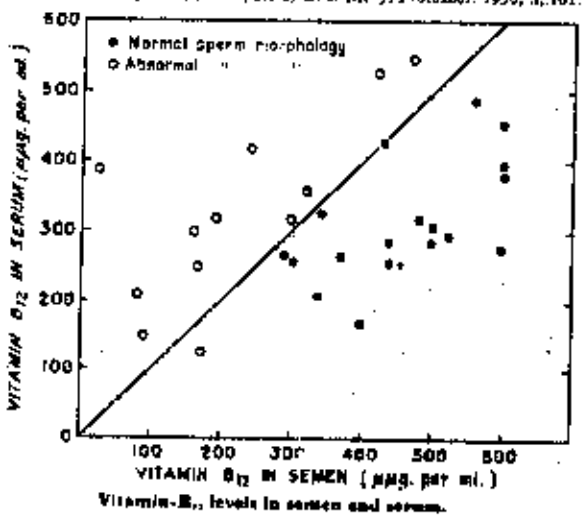

Vitamin-B_{12} levels in semen and serum.

CONCLUSION

These experimental findings suggest that vitamin B_{12} may influence the maturation of human spermatozoa.

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Intestinal Vitamin B_{12} Absorption in the Dog

III. Demonstration of the Intracellular Pathway of Absorption by Light and Electron Microscope Autoradiography

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The intracellular pathway of vitamin B_{12} absorption in the dog intestine *in vivo* was visualized by light and electron microscope autoradiography. The terminal ileum of each of five anesthetized mongrel dogs was divided by ligatures into separate 5-cm.-long *in situ* sacs; the lumen of each sac was washed thoroughly and filled with 0.75 μg. (~10 μc.) of $Co^{57}B_{12}$. At 30-minute, and 1-, 2-, and 3-hour intervals, the sacs were excised and prepared for autoradiography. During the initial 2-hour period following intraluminal radio-B_{12} instillation, label was localized principally in the surface mucus contained in crypts and attached to villi, and in mucus granules of goblet cells in both crypts and villi. At 3 hours, radioactivity was predominantly localized in mucosal absorbing cells and underlying lamina propria on light microscopy. On electron microscopy this radioactivity was localized in the area of the microvilli, terminal web, rough endoplasmic reticulum, Golgi apparatus, lateral cell membranes and intercellular spaces, lamina propria, and capillaries of the lamina propria. These results suggest that, at least in this species, goblet cell mucus B_{12} binders may play a role in the intestinal absorption of vitamin B_{12}. They also suggest that certain B_{12} transport proteins are synthesized within the endoplasmic reticulum of intestinal absorbing cells and are complexed with absorbed B_{12} during its passage through the cell before reaching the blood stream.

Additional key words: Pernicious anemia, Goblet cell, Mucus, Mucoproteins, Intestinal epithelium, B_{12} binders, Intrinsic factor.

Through numerous biochemical and physiologic studies, intestinal vitamin B_{12} (B_{12}) absorption in man and many animal species has been shown to proceed in several stages.[1-5] A physiologic dose of ingested B_{12} is first bound to intrinsic factor (IF) in the lumen of the gastrointestinal tract. IF is secreted by the stomach and is believed to be a glycoprotein with a molecular weight in the range of 50,000.

In the first stage of absorption following the binding of IF to B_{12}, the IF-B_{12} complex formed is accumulated or "adsorbed" onto IF-B_{12} receptors located along the surface of the intestinal mucosa. At this stage, the bond between IF-B_{12} and the mucosal surface is calcium-dependent and may be reversed by the addition of ethylenediaminetetraacetic acid. Following this stage of adsorption, B_{12} penetrates the mucosal absorbing cells, presumably by a process of active transport, and from there passes into the blood stream.

It is not yet known in what manner B_{12} is released from its bond to IF without damage to the vitamin, nor whether this occurs before or after its penetration into the mucosal cell. Once in the blood stream, however, intact B_{12} is no longer bound to IF but rather to the serum B_{12} binder, transcobalamin II.[7] In the guinea pig intestine, a further step in the absorption of B_{12} has been shown to consist of the binding of the IF-B_{12} complex to a macromolecular factor present in the ileum and termed ileal intrinsic factor.[8] Although the above process applies to absorption of the physiologic quantities of B_{12} normally found in food, a second mechanism exists for the absorption of supraphysiologic amounts of B_{12}, which is not IF-dependent and which is more likely one of passive diffusion.

Within the intestinal mucosa, little is known of the structural pathway followed by the B_{12} molecule during its absorption. In a brief report, the appearance of this absorptive process in the mucosa at one point in time was only recently described.[9] Under the light microscope, 1 hour after incubation of tritiated B_{12} and IF with segments of guinea pig ileum *in vitro*, radioactivity was observed in the brush border and apical portions of intestinal absorbing cells.

At the ultrastructural level, however, the intracellular pathway of the B_{12} molecule during its passage from intestinal lumen through the absorptive cell and into capillaries of the lamina propria is unknown. In the present investigation, therefore, light and electron microscope autoradiography were combined to visualize the details of this pathway in the dog intestine *in vivo*.

MATERIALS AND METHODS

Intestinal B_{12} absorption was studied in five fasting mongrel dogs by means of *in situ* ileal sacs.

Under pentobarbital anesthesia, the abdomen was opened surgically, the terminal ileum was identified, and double silk ligatures were placed at the ileocecal valve. The ileum was then divided into four to six 5-cm.-long ileal sacs by placing double silk ligatures at appropriate intervals proximal to the ileocecal valve. Care was taken to leave the entire mesentery intact and thereby to preserve the vascularization of the intestine.

The lumen of each sac was then washed three times with 5 to 7 ml. of warm neutralized 0.9 per cent saline injected transmurally. After washing, the contents of the sac were withdrawn, and 0.75 μg. (~10 μc.) of $Co^{57}B_{12}$ was instilled into the lumen of each sac by transmural injection. Following this injection, the intestine was carefully replaced into the abdomen, the abdominal wall was closed with hemostats, and the entire abdomen was covered with sterile towels which were kept continuously moist with warmed 0.9 per cent saline. At 15- and 30-minute and 1-, 2-, and 3-hour intervals, the abdomen was reopened by releasing the hemostats; one test sac was carefully mobilized and its mesentery was divided between two silk ligatures. The contents of the sac were first rapidly withdrawn and replaced with 5 ml. of phosphate-buffered glutaraldehyde[24]; the ends of the sac were then immediately cut between the double ligatures separating it from the remaining intestine. The removed sac was quickly opened and drained; a small tissue sample was excised from its center with a clean razor blade and placed in phosphate-buffered glutaraldehyde at room temperature. The remaining segment was washed five times with 10 mM $CaCl_2$, then blotted on filter paper, and weighed on a torsion balance. The segments were counted for radioactivity, and the tissue uptake of $Co^{57}B_{12}$ was calculated in picograms of B_{12} per gram wet weight of intestine. These results are included in separate reports.[27-29]

After 5 minutes in glutaraldehyde, the excised tissue sample was placed in a drop of glutaraldehyde solution on a pad of dental wax and was carefully divided into smaller blocks. These smaller samples were replaced in the glutaraldehyde solution for 1 hour and postfixed in 1 per cent phosphate-buffered osmium tetroxide[15] for 1 hour at room temperature. They were then dehydrated in graded alcohols and propylene oxide and embedded in Epon 812 according to the method of Luft.[14] Specimens were placed into embedding medium under a dissecting microscope or hand lens so that orientation of the specimen during embedment could be controlled.

Thick sections of 1 to 2 μ and thin sections displaying gold interference colors[19] were cut on a Huxley microtome with the use of glass knives. Sectioning was carried out in a well oriented specimen parallel to the plane of the intestinal villi. Thick sections were picked up on glass slides and processed for light microscope autoradiography by the dipping technique[13] with Kodak NTB2 or NTB3 emulsion, and then stored in light-tight black boxes for 3 to 8 weeks at 6° C. After exposure, the slides were developed for 2 minutes in a 1:3 solution of Dektol (Eastman Kodak Company, Rochester, New York),

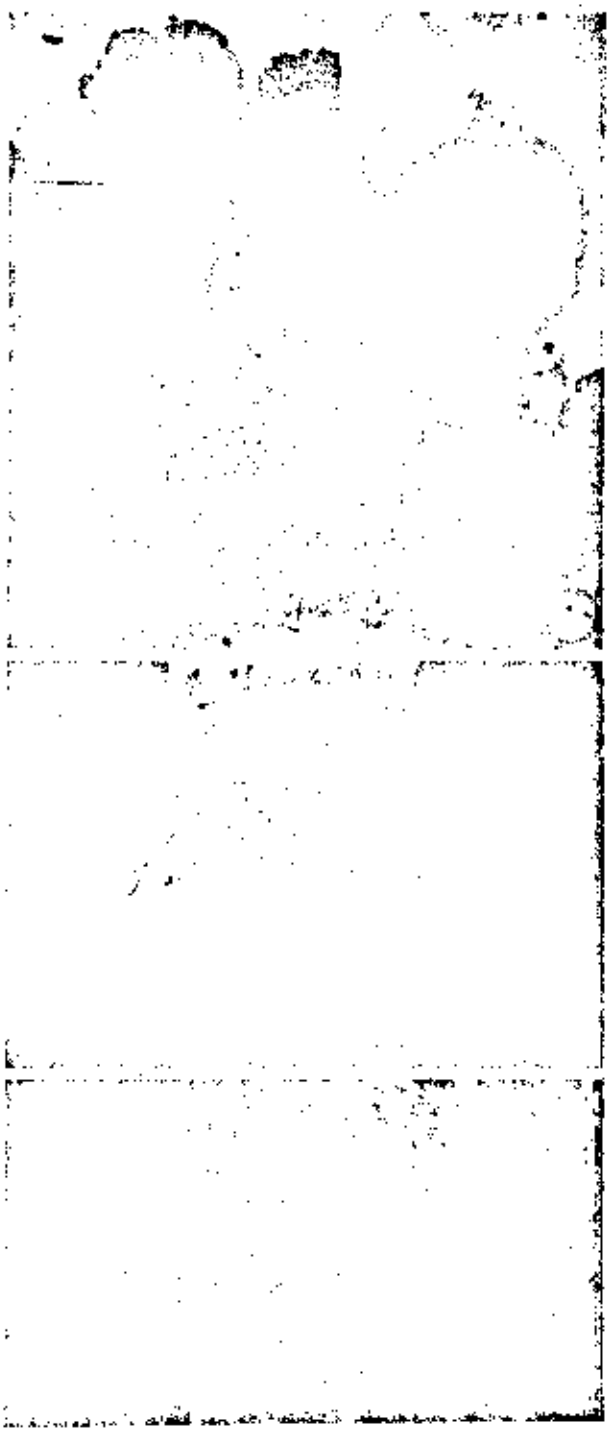


FIG. 1. Light microscope autoradiograph of specimen of dog ileum removed 2 hours following intraluminal instillation of radioactive vitamin B_{12}. Areas enclosed by *rectangles* are shown enlarged in Figures 2 and 3. ×75.

FIG. 2. Light microscope autoradiograph of villus area shown in Figure 1. Note concentration of developed grains overlying mucus fragment attached to villus and absence of grains over intestinal absorbing cells. ×200.

FIG. 3. Light microscope autoradiograph of crypt area shown in Figure 1. Concentration of developed grains overlies mucus content of crypt. ×200.

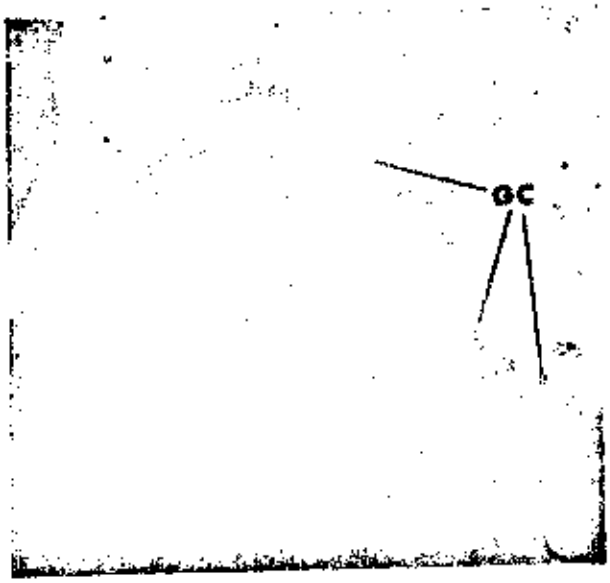


FIG. 4. Light microscope autoradiograph of ileum removed 2 hours following intraluminal instillation of radioactive vitamin B_{12}. Note concentration of developed grains over villus goblet cells (GG) and the absence of grains over intestinal absorbing cells. ×160.

FIG. 5. Light microscope autoradiograph of ileum removed 5 hours following intraluminal instillation of radioactive vitamin B_{12}. Developed grains now overlie intestinal absorbing cells and lamina propria. ×200.

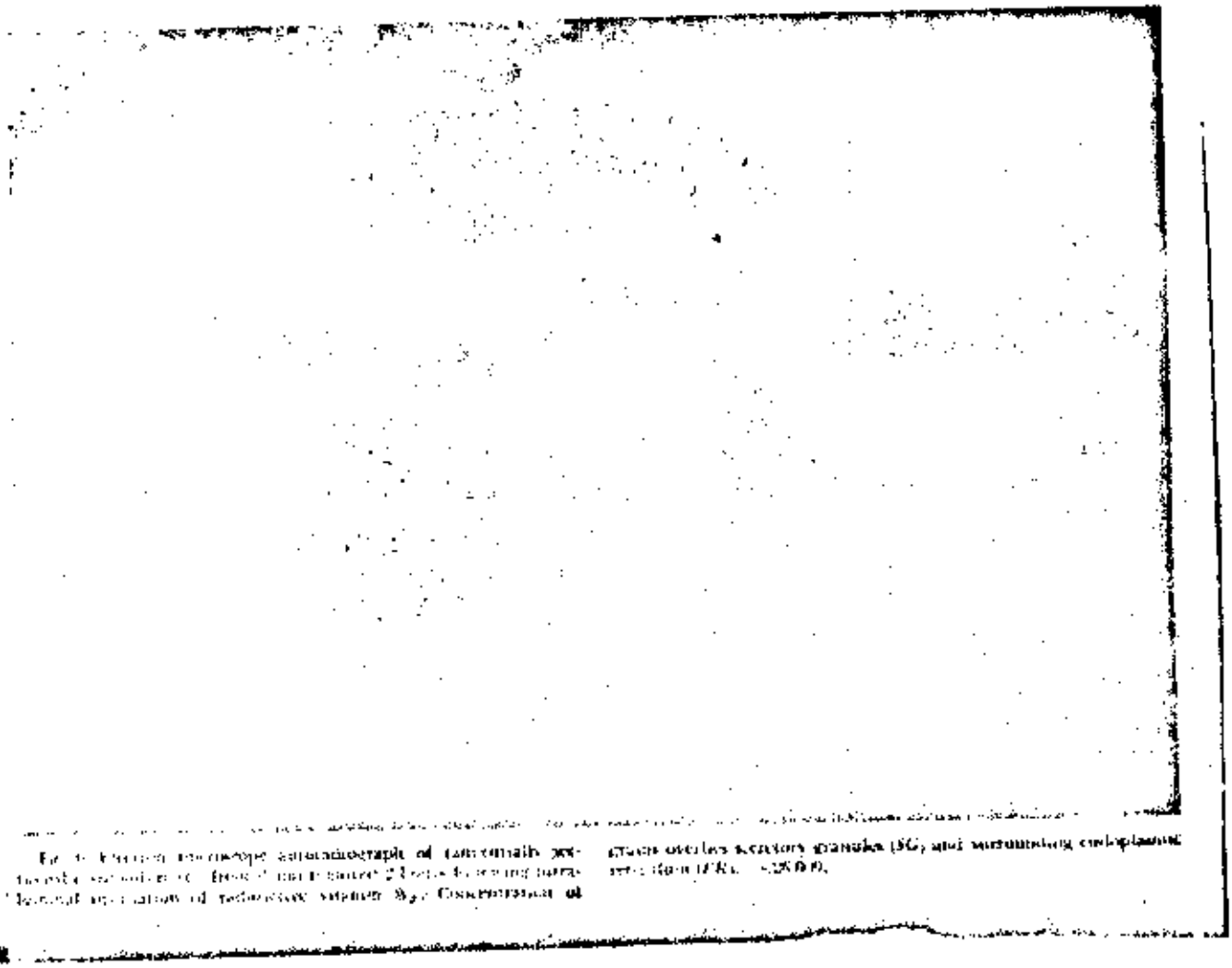


FIG. 6. Electron microscope autoradiograph of intestinal absorbing cell from ileum removed 2 hours following intraluminal instillation of radioactive vitamin B_{12}. Concentration of silver grains overlies secretory granules (SG) and surrounding cytoplasmic matrix (CM). ×25,000.

wa ed, and fixed in acid fix for 10 minutes. After a sec- oc washing, the sections were stained with toluidine bl and examined.

Thin sections were picked up on Formvar-coated 75-me copper grids and were coated with Ilford L-4 emulsion according to the method of Koehler, Mühlethaler, and Freywyssling,[19] with minor modifications. Coated grids were stored for 3 to 6 months in black light-tight boxes at 4° C. After exposure, grids were developed in a 1/2 solution of Kodak D-19, then fixed in acid fix, double stained with uranyl acetate[25] and lead citrate,[22] and examined in a Siemens-Elmiskop IA electron microscope.

RESULTS

Light Microscope Autoradiography

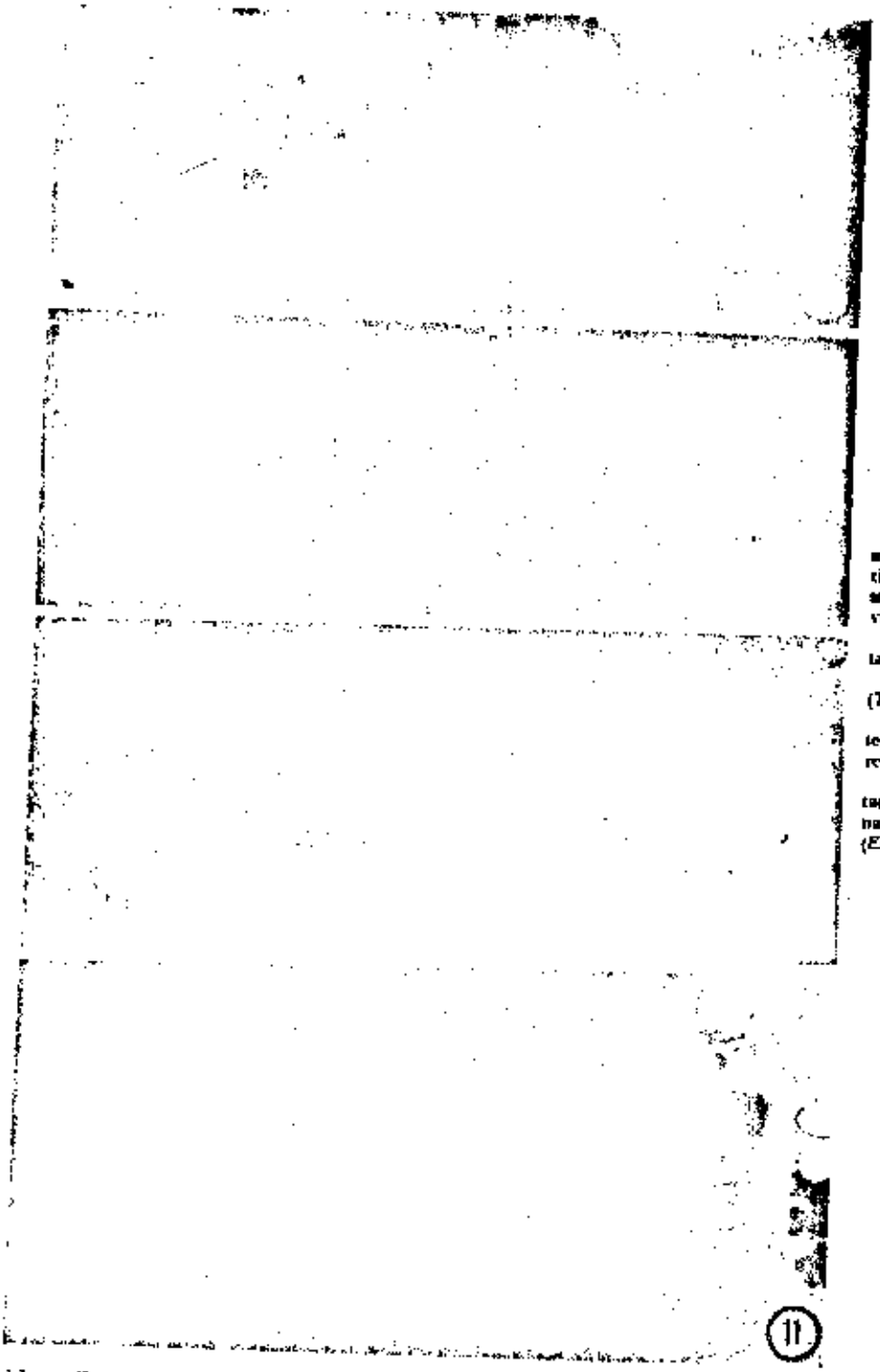
Autoradiographs of thick sections taken from specimens during the first 2 hours after instillation of radio-B_{12} are shown in Figures 1 to 4. At this time, radioactivity was localized in the mucous contents of crypts and in the fragments of mucus attached to villi (Figs. 2 and 3). Radioactivity could also be seen within goblet cells in both crypts and villi (Fig. 4), but practically no radioactivity was seen during this period within villous absorbing cells (Figs. 3 and 4).

At 3 hours, the distribution of radioactivity within the intestine changed. Considerable radioactivity was now localized within the absorbing cells of the villus and in the underlying lamina propria although some radioactivity was still present within goblet cells and crypts (Fig. 5).

Electron Microscope Autoradiography

During the first 2 hours after instillation of radio-B_{12}, the most striking finding at low magnifications was the frequent accumulation of radioactivity within goblet cells of both crypts and villi, seen previously with the light microscope. At higher magnifications, however, it was additionally clear that the radioactivity was localized in the secretory granules of the goblet cell (Figs. 6 and 7). Occasionally, collections of radioactivity could also be seen within channels of endoplasmic reticulum surrounding the central mass of mucous granules (Fig. 6). In vertical sections through the apical pole of some

[illegible] autoradiograph of a vertical section [illegible] of cells of a villus-goblet cell from a dog removed 2 [illegible] after instillation of radioactive vitamin B_{12}. Concentration of grains overlies secretory granules (SG) and discharged mucus. ×21,000.



Note: Figures 8 to 11 are electron microscope autoradiographs of intestinal absorbing cells from ileum removed 3 hours following radioactive vitamin B_{12} instillation.

FIG. 8. Grains overlie mucus attached to microvilli (*MV*). ×15,000.

FIG. 9. Grains overlie terminal web (*TW*). ×32,000.

FIG. 10. Grains overlie microvilli, terminal web (*TW*), and endoplasmic reticulum (*ER*). ×33,600.

FIG. 11. Grains overlie mucus attached to microvilli, fuzzy layer, terminal web, and endoplasmic reticulum (*ER*). ×9,300.

goblet cells, radioactivity could be seen attached simultaneously to dissolved mucus outside the cell and to mucous granules within the cell (*Fig. 7*). It is therefore apparent from these micrographs that radio-B_{12} instilled intraluminally is not only attached to mucus distributed over the surface of the intestine but also to the mucus within goblet cells.

Autoradiographs of ileal sections taken 3 hours after intraluminal instillation of radio-B_{12} confirmed the presence of radio-B_{12} within absorbing cells of the villi (Figs. 8 to 14). The principal sites of localization for radio-B_{12} at this time were the mucus attached to microvilli (Fig. 8), the area of the fuzzy layer and microvilli (Figs. 10 and 11), the terminal web (Figs. 9 to 12), the Golgi apparatus (Figs. 13 and 14), and along lateral cell membranes and in intercellular spaces (Figs. 15 and 16). From

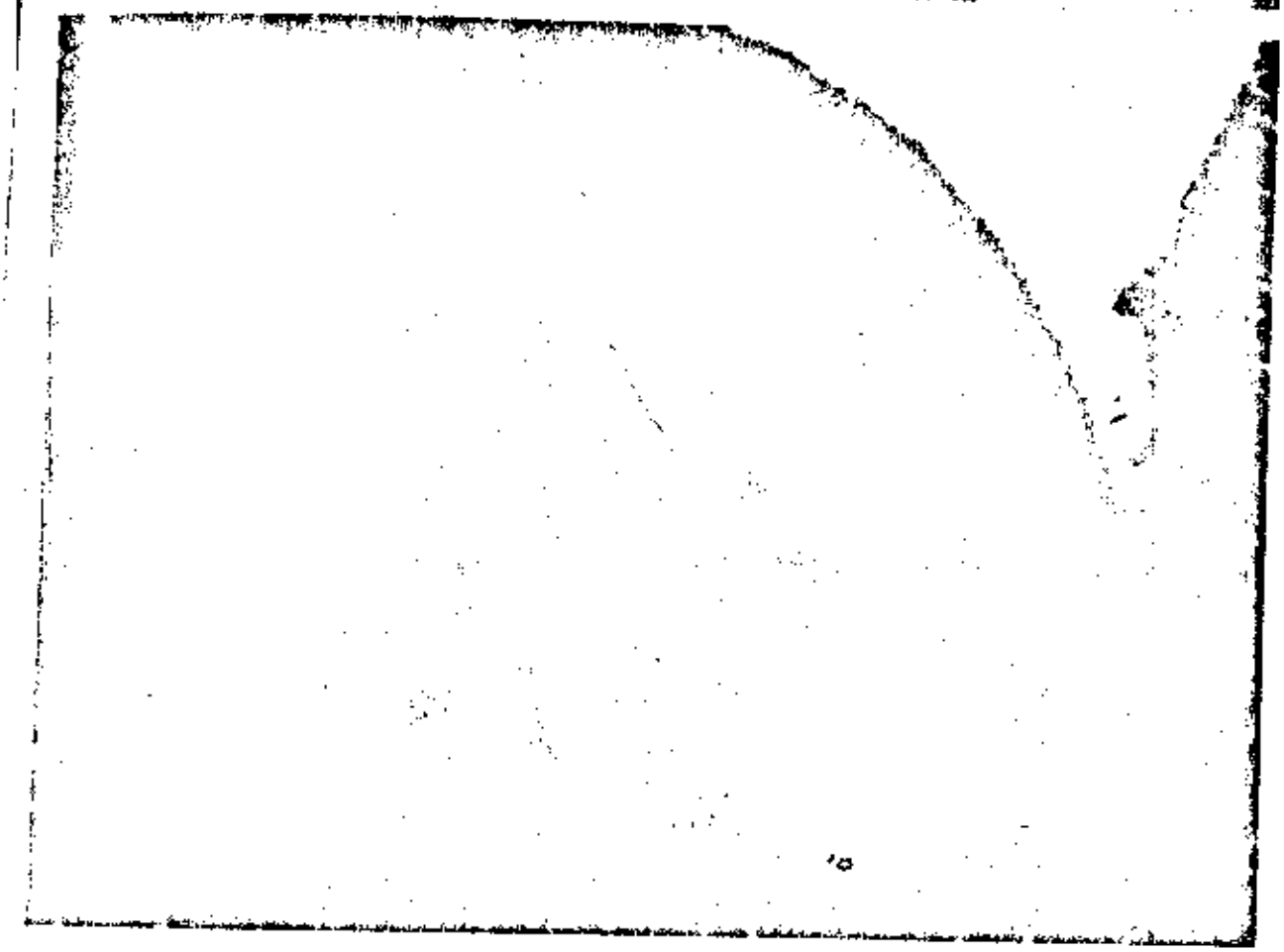


FIG. 12. Electron microscope autoradiograph of intestinal absorbing cell from ileum removed 3 hours following intraluminal instillation of radioactive vitamin B_{12}. Developed grains overlie terminal web (*TW*) and channels of the endoplasmic reticulum (*ER*). ×38,000.

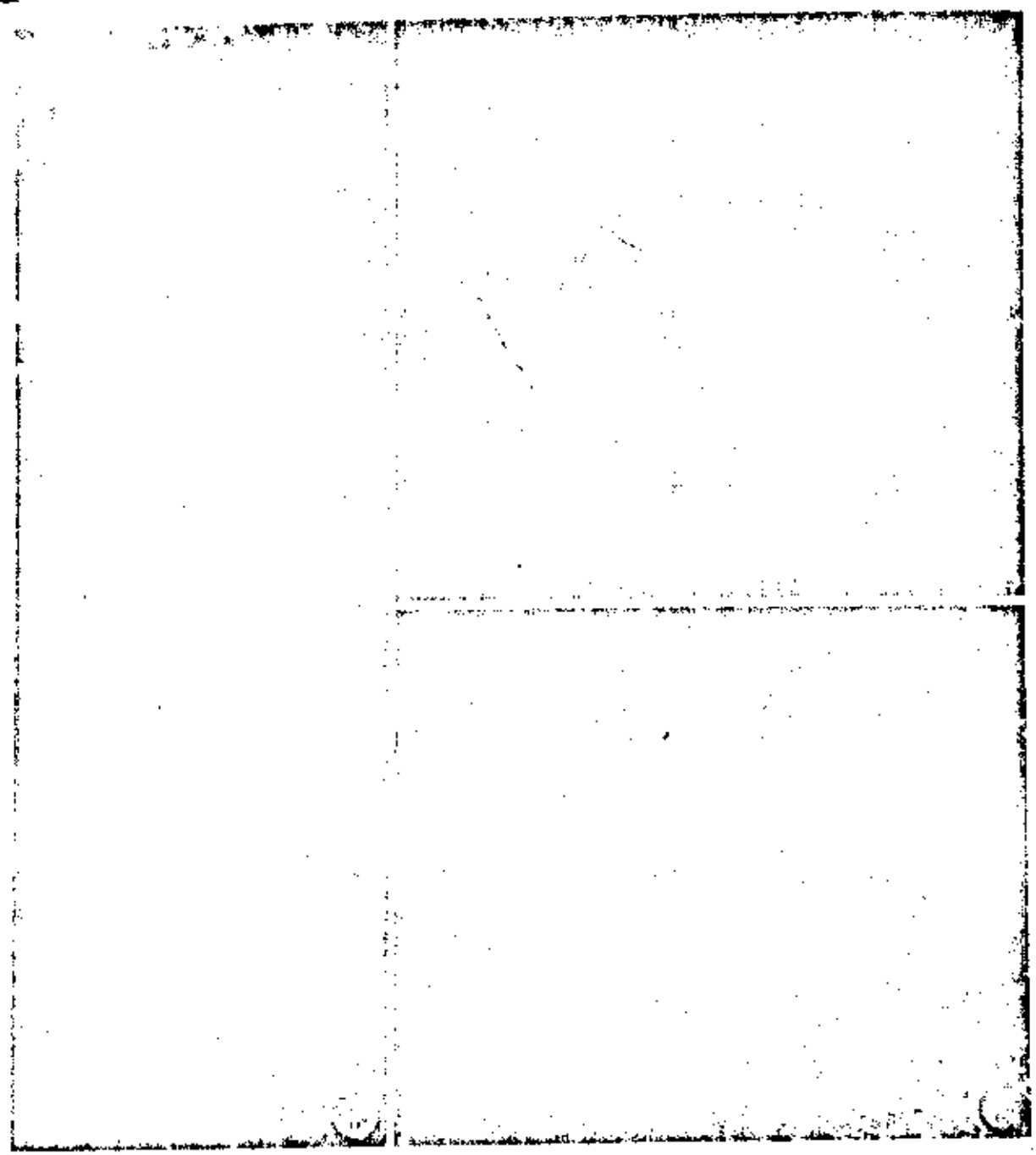
these spaces the label appeared to pass between adjacent cells, through the basal lamina (Fig. 16) and into the capillaries of the lamina propria (Figs. 16 and 17). Within capillary walls it was found most frequently over the numerous fenestrae which punctuate the endothelial cell cytoplasm (Figs. 16 and 17). A quantitative determination of the frequency distribution of developed grains overlying intracellular sites in 80 well oriented intestinal absorbing cells is shown in Table 1.

In the region of the microvillous border (Figs. 10 and 11), the large size of developed grains precluded the precise localization of radioactivity within or between adjacent microvilli because in each instance a single grain always overlapped both the microvillous and intermicrovillous space. At points of entrance (microvillous border and terminal web area) and exit (lateral cell membranes) to and from the cell, no specific ultrastructural unit, either vesicle or tubule, could be seen to transport label into or out of the cell. Finally, in the lamina propria no accumulation of radioactivity within lymphatic channels was seen.

DISCUSSION

The present study has described in ultrastructural detail the intracellular pathway of intestinal vitamin B_{12} absorption in the dog. This pathway is illustrated schematically in Figure 18.

The most striking finding was the early localization of radio-B_{12} in the mucus contained in crypts, attached to villi, and in the secretory granules of goblet cells in both crypts and villi. This phase of attachment of B_{12} to intestinal goblet cell mucus corresponds in time to the initial phase of intestinal B_{12} absorption described in biochemical investigations and termed "surface adsorption" by Okuda.[14] During this phase, B_{12} adheres to the intestinal mucosal surface by a calcium-dependent bond which may be dissolved by the addition of ethylenediaminetetraacetic acid. Since a number of mucoproteins contain calcium bonds linking amino and carboxyl groups which are susceptible to the action of ethylenediaminetetraacetic acid,[12] the attachment of B_{12} to intestinal mucus described in the present study may be the morphologic equivalent of the surface adsorption described in biochemical work.[5, 14]



FIGS. 13 AND 14. Electron microscope autoradiographs of two intestinal absorbing cells from ileum removed 5 hours following radioactive vitamin B_{12} instillation showing concentrations of developed grains over the Golgi region (*G*). Figure 13, ×18,000; Figure 14, ×25,000.

FIG. 15. Autoradiograph of basal portion of intestinal absorbing cells and lamina propria from ileum removed 3 hours following radioactive vitamin B_{12} instillation. Developed grains overlie lateral cell membranes (*LCM*) above the basement membrane (*BM*). ×24,000.

The importance of the preliminary binding of B_{12} to mucus during the process of absorption is suggested by the recent findings of Rothenberg.[23] In his studies on B_{12} absorption in the guinea pig ileum, Rothenberg has shown that a step in B_{12} absorption involves the binding of IF-B_{12} complex to a macromolecular factor, probably a protein, present in ileal mucosa. It may be that this is a mucoprotein of goblet cell origin. If true, this would point to a role for the goblet cell component of intestinal mucus in one phase of the absorption of some nutrients.

It is not certain, however, that all of the mucus

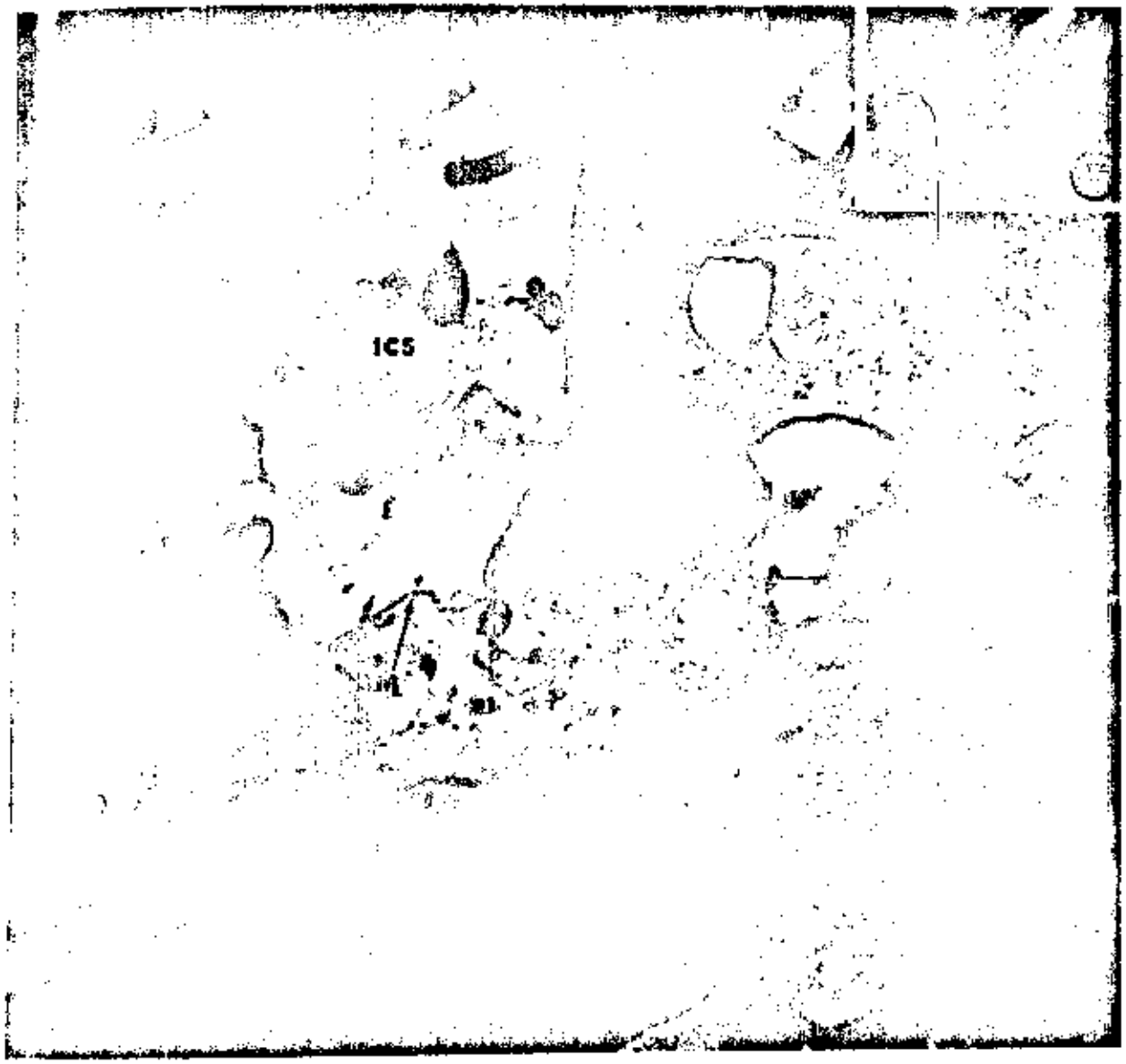


FIG. 16. Autoradiograph of basal portion of intestinal absorbing cells and lamina propria from ileum removed 3 hours following radioactive vitamin B_{12} instillation. Developed grains overlie intercellular space (*ICS*), basement membrane (*BM*), and fenestrated portion of capillary endothelial cell cytoplasm. ×24,000.

FIG. 17. Autoradiograph of lamina propria capillary 3 hours following radioactive vitamin B_{12} instillation showing developed grain overlying fenestration in capillary endothelial cell cytoplasm. ×9,000.

found radio-B_{12} observed in the present study represents B_{12} that subsequently enters intestinal absorbing cells. In the rat, at least two kinds of surface adsorption have been described,[19] one representing a phase of the process of physiologic absorption and the other a nonspecific type mediated by non-IF mucoproteins, not part of the absorptive process. In the dog, on the other hand, the significance of the adsorptive phase may be different from that in the rat or in other species in which B_{12} absorption is IF-dependent. In parallel studies in the rat[19,20] we have been unable to identify IF in the stomach and duodenum nor to find any evidence that luminal B_{12} absorption is IF-dependent in this species.

At 3 hours after intraluminal instillation of radio-B_{12} alone, radioactivity was localized, by light microscope autoradiography, to intestinal absorptive cells and underlying lamina propria. Since the radio-B_{12} instilled was not bound to IF and since the intestinal sacs had been thoroughly washed prior to radio-B_{12} instillation, these results support our previously reported findings[27-29] that physiologic B_{12} absorption in the dog intestine may proceed in the absence of IF.

At the ultrastructural level, although the absorptive pathway was seen to commence at the microvillous surface of the mucosal cells, the large size of developed grains precluded their precise localization within or be-

tween microvilli. There is indirect evidence at the present time, however, which indicates that the microvillous membrane does participate in the B_{12} absorptive process.[8]

Beneath the microvilli, radio-B_{12} was localized to the terminal web area and was not associated with any underlying tubular or vesicular system. In particular, no pinocytotic vesicles were seen regularly associated with developed grains. Within the limits of the technique of fixation and preservation used here, this indicates that the B_{12} molecule and its carrier protein are moving through dense cellular matrix before reaching channels of endoplasmic reticulum.

The absence of pinocytotic vesicles in the terminal web area associated with radio-B_{12} absorption is somewhat at variance with an earlier report[17] in which maximal B_{12} absorption in the visceral yolk sac of the rat *in vitro* was associated with a free surface appearance characteristic of a pinocytotic membrane. The uptake of radio-B_{12} by pinocytotic vesicles, however, was not directly demonstrated in that study. It is also not certain whether the visceral yolk sac of the rat and the adult dog intestine absorbs nutrients in a comparable manner.

Beneath the terminal web area, radio-B_{12} was localized principally in rough endoplasmic reticulum, Golgi apparatus, and intercellular spaces. This pathway has also been described for fat absorption, with the exception of a somewhat more prominent role of the smooth surfaced endoplasmic reticulum during that process.[1, 12, 19, 20] The results of this study as well as those for fat absorption indicate that this general intracellular route is a fundamental one which may be shared by a variety of nutrients.

This pathway is also consistent with some recent biochemical observations. During passage through the rat intestinal mucosa *in vivo*, the B_{12} molecule has been shown, by fractionation on Sephadex G-100 columns, to be bound to proteins with a molecular size of 100,000 or greater before the molecule undergoes final attachment to serum proteins and subsequent removal by the circulation.[4] Because the endoplasmic reticulum and Golgi apparatus are known sites of protein synthesis, these B_{12} "transport" proteins are probably synthesized within endoplasmic reticulum and Golgi channels and complexed with absorbed B_{12} as it passes through them. Although the role of these proteins in the transport of B_{12} is not completely understood, Gräsbeck[8] has recently suggested that the serum binder transcobalamin II, to which B_{12} absorbed from the intestine is normally found attached in the blood, is synthesized within the intestinal cell. Their role may thus be analogous to that of the low density lipoproteins which are synthesized during fat absorption within intestinal cells and which then participate in the formation of chylomicrons, the form in which absorbed fat is transported in the blood.[9]

In its final phase, the absorptive process consisted of the movement of radio-B_{12} from lateral intracellular spaces directly through the basement membrane into the lamina propria and capillaries of the lamina propria. During its passage through capillary walls, developed grains were localized most frequently over the numerous fenestrations which characterize the intestinal capillary endothelium in this species. These fenestrations seem, therefore, to function as sites of entry for nutrients absorbed into the circulation. No appreciable radioactivity was seen over lymphatic channels, which is in line with earlier work[21] indicating that intestinal B_{12} absorption in the dog proceeds mainly by way of the portal circulation.

In conclusion, it must be emphasized that the present study has visualized the intracellular pathway of in-

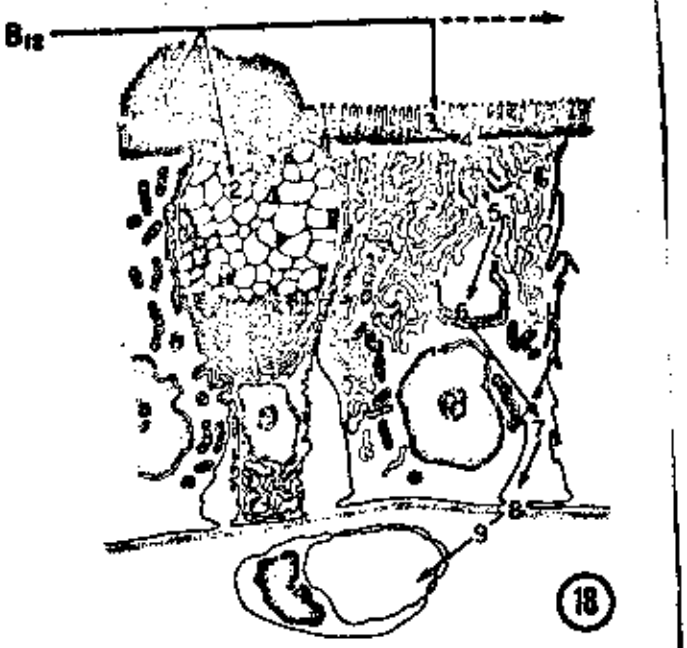


FIG. 18. Schematic illustration of the ultrastructural pathway for vitamin B_{12} absorption in the dog intestine. B_{12} is first bound to surface mucus (*1*) and to mucus granules within goblet cells (*2*). B_{12} then penetrates the intestinal absorptive cell, moving through the area of the microvilli (*3*), terminal web (*4*), rough endoplasmic reticulum (*5*), and Golgi apparatus (*6*). It then leaves the cell via the lateral intercellular spaces (*7*), penetrates the basement membrane (*8*) into the lamina propria (*9*), and enters capillaries of the lamina propria across their fenestrated endothelium. Interrupted arrow indicates the possibility that some mucus-bound B_{12} is excreted unabsorbed.

TABLE 1. FREQUENCY DISTRIBUTION OF DEVELOPED GRAINS OVERLYING INTRACELLULAR SITES IN 80 WELL ORIENTED INTESTINAL ABSORBING CELLS

Cell constituent	No. of grains	% of total grain count
Microvilli	30	18
Terminal web	62	22
Endoplasmic reticulum	69	25
Golgi apparatus	35	13
Lateral cell membranes (and intercellular spaces)	49	18
Lysosomes	1	0.1
Nuclei	3	2
Mitochondria	8	3

test ... al B_{12} absorption in a species in which the process of ... absorption does not appear to be IF-dependent.[27-... is by no means certain whether the same ultrastr... tural sequence would hold for other species in wh... physiologic absorption is IF-dependent, nor wh... her it would be the same necessarily for supraphy...iologic doses of B_{12}.

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BRIEF REPORT

Vitamin B_{12} and Folate Activity in Normal Human Platelets

By Harvey J. Weiss, Alan Kelly and Victor Herbert

ALTHOUGH the vitamin B_{12} and folate contents of human erythrocytes and leukocytes have been established, information regarding the content of these vitamins in normal human platelets is lacking. The results of studies to determine these values are presented here.

Methods

Preparation of platelets. Venous blood from normal male and female subjects, ages 20–4[illegible] was mixed (8:1) with 1 per cent EDTA in 0.7 per cent saline in siliconized tubes and centrifuged at 2500 rpm in a PR-2 centrifuge for 3 minutes to obtain platelet-rich plasma (PRP). Subsequent centrifugations were at 3000 rpm and 4 C. for 30 minutes. A platelet button was obtained by centrifuging the PRP, and the platelets were washed 7 times with one-half the plasma volume of cold .05M imidazole-buffered isotonic saline, pH 7.3, containing 0.1 per cent EDTA, in siliconized, graduated McNaught tubes. Where necessary, contaminating red cells were removed during the washings.

Vitamin B_{12} studies. The washed, packed platelets from each of 16 normal subjects were suspended in isotonic saline in a final concentration of 1 per cent (V/V) and the platelets counted by phase microscopy. The suspension contained no leukocytes and 0–30 red cells per 10^8 platelets. In order to extract vitamin B_{12} in a soluble form suitable for microbiological assay, the platelets were disrupted by ultra-sonic vibration. This was achieved by sonicating 2 ml. of the platelet suspension, immersed in ice, for 5 minutes at maximal amplitude.* After this procedure, no platelets or platelet fragments were seen by phase microscopy. The sonicate was then centrifuged at 24,000 g for 30 minutes at 4 C. and the supernatant removed and stored for 2–10 days at −20 C. Vitamin B_{12} activity in the supernatant was assayed microbiologically, in a 1:10 flask dilution, using *Euglena gracilis* as the test organism† and the B_{12} activity in platelets expressed as either pg per 10^9 cells or pg per ml. of packed platelets.

Folate. Washed, packed platelets were suspended, in a 1 per cent V/V concentration, in 0.05 M phosphate buffer, pH 6.2, containing 0.5 per cent ascorbate. The suspensions were sonicated, centrifuged and the supernatants stored as described for B_{12} assay. Folate

*Edison Ultrasonic Disintegrator (20 KC, 200 W.), Edison Instruments, Inc., Rahway, New Jersey.

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Table 1. — *Vitamin B_{12} and Folate Values*

Subject	Vitamin B_{12}			Folate		
	pg. per ml. packed plts.	pg. per 10^9 plts.	pg. per ml. serum	ng. per ml. packed plts.	ng. per 10^9 plts.	ng. per ml. serum
1	2100	5.5	341	70	0.18	11.6
2	1000	2.8	451	60	0.17	8.5
3	1100	—	—	40	—	9.8
4	1600	—	345	80	—	7.7
5	1000	2.8	542	60	0.16	—
6	1200	3.2	209	50	0.13	5.7
7	1400	—	402	170	—	13.6
8	1600	5.6	430	40	0.14	5.5
9	1200	3.7	288	40	0.12	8.5
10	1100	3.1	301	60	0.16	8.7
11	1800	2.9	650	70	0.17	15.6
12	1100	—	—	—	—	—
13	1900	5.7	—	—	—	—
14	1100	2.7	—	—	—	—
15	1100	2.4	—	—	—	—
16	600	1.2	—	—	—	—
Mean	1288	3.5		67	0.15	
S.D.	372	1.4		36	.02	
Range	600–2100	1.2–5.7		40–170	0.11–0.18	

activity in the supernatants was assayed microbiologically using *Lactobacillus casei*.[3] In order to obtain values which could be read from the linear portion of the standard curve, 1.0 ml. aliquots were assayed and results were expressed as ng. per ml. of packed cells. Failure to add ascorbate to the suspension media resulted in markedly lower folate activity, as previously reported for serum.[3] Platelets underwent marked clumping in the ascorbate buffer so that it was not possible to count them. To estimate the platelet folate content, the number of platelets in the 1 per cent ascorbate suspension for each subject was assumed to be the same as that in the 1 per cent saline suspension prepared on the same day for B_{12} determination.

Results

Vitamin B_{12}

Total B_{12} activity (Table 1). The mean platelet B_{12} concentration in 16 normal subjects was 1288 pg. per ml. of packed cells, with a range of 600–2100. The mean platelet B_{12} content, calculated from the platelet count of the 1 per cent suspension, was 3.5 pg. per 10^9 platelets, with a range of 1.2–5.7. There was no correlation between serum and platelet B_{12} activity ($p > 0.10$). In 2 patients with Glanzmann's thrombasthenia, platelet B_{12} concentration was normal (1100 and 1500 pg. per ml. packed cells).

Folate

(a) In 11 subjects, the mean platelet folate concentration was 67 ng. per ml. of packed platelets, with a range of 40–170. The mean folate content was 0.15 ng. per 10^9 platelets, with a range of 0.11 to 0.18. The linear correlation between serum and platelet folate was significant at the .10, but not the .05, level of probability ($[r]_8 = .553$).

(b) Effect of incubating platelets with plasma prior to assay: platelets were washed 7 times, suspended in a 1.5% (V/V) concentration in ascorbate buffer

Table 2.—*B_{12} and Folate Values in Blood Components*

	B_{12}		Folate	
	pg/ml packed cells	pg/10^9 cells	ng/ml packed cells	ng/10^9 cells
Platelets	1268 (600–2100)	3.5 (1.2–5.7)	97 (40–170)	0.15 (.11–.18)
Red Cells	205[4] (72–512)	1.27[4†]	316[5] (166–840)	2.6[5†]
White Cells	7660[6*]	384[6] (100–800)	547[14] (263–1028)	27.3[13]
	7920[6*]	396[6] (245–665)		
Serum	472[7] (103–925)		10[3] (5–25)	

*Calculated from values reported as pg/10^9 cells on the assumption that 0.50 ml of packed leukocytes contain 10^9 cells.[14]

†Calculated from values reported per ml of packed cells on the assumption that 1.0 ml packed red cells contain 1.2×10^{12} cells.

Figures shown denote mean and range of values.

and sonicated. 2.5 ml. of the sonicate was incubated with 0.04 ml. of platelet-free plasma from a normal subject at 37 C for 90 minutes. The ratio (V/V) of plasma to platelets in the incubation mixture was $\frac{.04}{2.5 \times .015} = 1.17$. As a control, platelet sonicate was incubated with saline. The samples were then centrifuged at 24,000 g for 45 minutes and the supernatants assayed for folate activity. The contribution of the plasma, determined separately, was subtracted and platelet folate activity calculated. The results showed that incubation of the platelet sonicate with plasma does not increase the measured folate activity.

Values Obtained in Extracts Prepared by Freeze-Thawing

Platelet suspensions from 3 subjects were either sonicated or frozen and thawed 3 times, using a dry ice-acetone mixture, and then centrifuged to obtain extracts. Sonication was 1.5–2 times as effective as freeze-thawing in disrupting platelets, as determined by the amount of protein present in the extracts. Extracts obtained by freeze-thawing contained an average of 80 per cent of the B_{12} activity and 125 per cent of the folate activity found in those obtained by sonication.

Discussion

The B_{12} concentration in platelets is about sixfold that in the erythrocyte,[4] one-sixth that in the leukocyte,[6,8] and 3 times that in serum[7] (Table 2). The contribution of platelets to whole blood B_{12} activity would, however, be small under normal circumstances. In whole blood whose platelet count was 300,000 per cu. mm., the B_{12} activity contributed by platelets would be 6–21 pg. per ml.

In contrast to B_{12}, the concentration of folate activity in platelets is only

⅙ that in red cells[8] (Table 2). In whole blood whose platelet count was 300,000 per cu. mm., the folate activity contributed by platelets would be 0.4–1.7 ng. per ml. Toennies et al.[9] reported that the measured folate activity in erythrocytes was strikingly increased if the cells were incubated with plasma prior to disrupting them by osmotic lysis. This was confirmed by Hoffbrand et al.[8] Toennies et al. also found that the folate activity of stromal-free red cell extracts was increased by incubation with plasma and postulated the existence of a plasma factor which converted red cell folate to a more active form.[9] In the present study, no such plasma activation could be demonstrated for the folate present in platelet extracts.

B_{12} and folate activity can only be assayed as soluble vitamins and, hence, it was not possible to assay the small pellet which remained after the sonication procedure. It is possible, therefore, that the actual platelet B_{12} and folate contents are higher than those reported here, a reservation which would also apply to previous studies in which these vitamins had been extracted from cells and tissues.

In human folate and B_{12} deficiency, large megakaryocytes with multisegmented nuclei are often seen. The thrombocytopenia which may occur in these conditions is presumably the result of impaired megakaryocyte maturation due to defective DNA synthesis, similar to the maturation defect in erythrocytes and leukocytes. Like the mature red cell, the circulating platelet contains no DNA and only small amounts of RNA[10] and lacks, therefore, the major requirement for both B_{12} and folate. As in the red cell, however, the platelet contains both vitamins in appreciable amounts. Whether they serve any useful function in the circulating platelet is not clear. In pernicious anemia, the circulating platelet may be functionally abnormal.[11,12] The recent report that platelets are capable of protein synthesis also suggests a B_{12} and folate requirement.[13,14]

Summary

The vitamin B_{12} and folate content of human platelets have been determined. The B_{12} concentration was sixfold that in red cells and one-sixth that in leukocytes. In normal whole blood, with a platelet count of 300,000 per cu. mm., the B_{12} activity contributed by platelets would be 6–21 pg. per ml. The contribution of platelets to the folate activity of normal whole blood averaged 0.4–1.7 ng. per ml. The folate activity in platelets was one-fifth that in an equal volume of red cells, but unlike red cell folate, was not increased by incubating platelet extracts with plasma.

SUMMARIO IN INTERLINGUA

Esseva determinate le contento de vitamina B_{12} e de folato in plachettas human. Le concentration de B_{12} esseva sex vices illo trovate in erythrocytos e un sexte illo in leucocytos. In normal sanguine total—con un numeration de plachettas de 300,000 per mm^3—le contribution de plachettas al activitate total de B_{12} esseva inter 6 e 21 pg per ml. Le contribution del plachettas al activitate folate in normal sanguine total esseva inter 0,4 e 1,7 ng per ml. Le activitate de folato in le plachettas esseva un quinto de illo trovate in le mesme volumine de erythrocytos. Tamen, per contrasto con lo que vale pro folato erythrocytic, le folato plachettal non esseva augmentate per le incubation de extracto plachettal con plasma.

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Biochemical Role of Vitamin B_{12}[1]

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FROM THE FIRST REALIZATION that pernicious anemia can be cured by dietary supplementation (77), considerable interest has centered on the active therapeutic principle. The isolation of vitamin B_{12} [5,6-dimethylbenzimidazolylcobamide cyanide (cyano-B_{12})] in 1948 (99, 105, 137) and the subsequent elucidation of its structure (49) provided the groundwork for studies on the mechanism of action of this vitamin.[3] That the vitamin was functioning in biological reactions in the form of a coenzyme, analogous to the situation seen with other vitamins of the B group, seemed quite probable, especially in light of the minute levels of the vitamin needed to satisfy nutritional requirements.

The isolation of deoxyadenosyl-B_{12}, the first coenzyme form of cyano-B_{12} (Fig. 1), by Barker and his group (3, 5, 6) proved this postulate and initiated a new phase in the understanding of the role of cyano-B_{12} in metabolism.

This review attempts to summarize recent studies on the biochemistry of deoxyadenosyl-B_{12} and related derivatives. After a brief introduction, the chemistry of these compounds and their role in enzymatic reactions and animal physiology are discussed.

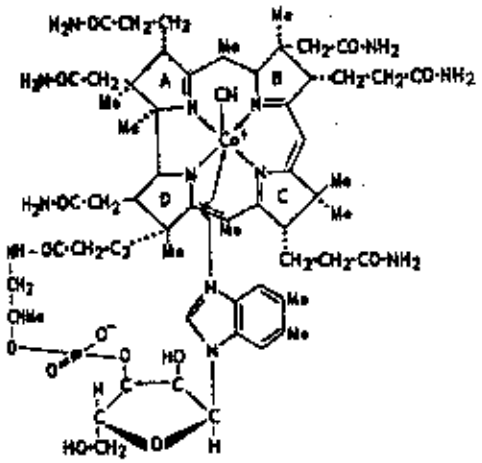
ISOLATION, STRUCTURE, AND OCCURRENCE

Studies on the fermentation of glutamic acid by cell-free extracts of *Clostridium tetanomorphum* (4, 197) led to the isolation of the first coenzyme form of cyano-B_{12} (5). Glutamate is converted by a series of reversible reactions to acetate and pyruvate. The initial reaction in this sequence involves the isomerization of glutamate to β-methylaspartate. Treatment of the crude enzyme preparation with

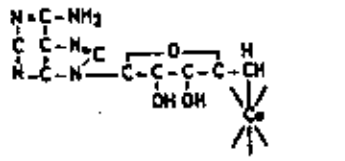
[1] This review does not include work published after January 31, 1964. It should also be noted that several excellent reviews on vitamin B_{12} have appeared in recent years which may be of help to the reader. These include: Beck, W. S., *New Engl. J. Med.*, 268: 708, 765, 814, 1963; Glass, G. B. J., *Physiol. Rev.*, 43: 529, 1963; Vitamin B_{12} Coenzymes, *Ann. N. Y. Acad. Sci.*, April 1963.

[2] Established Investigator of the American Heart Association, Inc.

[3] The following abbreviations are used: cyano-B_{12}: 5,6-dimethylbenzimidazolylcobamide cyanide; B_{12r}: a reduced form of the vitamin having cobalt in a valence state of 2; hydrido-B_{12}: a 2-electron reduced derivative of the vitamin; factor B: the vitamin minus the nucleotide; deoxyadenosyl-B_{12}: 5,6-dimethylbenzimidazolylcobamide 5′-deoxyadenosyl; methyl-B_{12}, ethyl-B_{12}, etc.: 5,6-dimethylbenzimidazolylcobamide methyl, ethyl, etc; AMe: S-adenosylmethionine; methyl-folate-H_4: N^5-methyltetrahydrofolic acid; FIGLU: formiminoglutamic acid.



Vitamin B_{12}
(Cyano-B_{12})



Coenzyme B_{12}(deoxyadenosyl-B_{12})

FIG. 1. Structure of vitamin B_{12} (cyano-B_{12}) and a schematic representation of the structure of coenzyme B_{12} (deoxyadenosyl B_{12}). The coenzyme contains a 5′-deoxyadenosyl group attached to the cobalt in place of the cyanide moiety of the vitamin. Other alkyl-B_{12} derivatives (methyl-B_{12}, ethyl-B_{12}, etc.) differ from the vitamin in that they contain the alkyl moiety in place of cyanide. Hydroxy-B_{12} contains a hydroxyl group in place of cyanide. B_{12a} differs from the vitamin in that the cobalt is in the divalent (not trivalent) state (reduced by 1 electron) and hydrido-B_{12} is thought to contain monovalent cobalt (reduced by 2 electrons). The series of compounds related to adeninylcobamide-cyanide contain adenine in place of 5,6-dimethylbenzimidazole in the vitamin structure. Factor B lacks the entire nucleotide (5,6-dimethylbenzimidazole-ribose-PO_4).

charcoal (5) resulted in loss of isomerase activity, which could be restored by the addition of a boiled cell extract, an indication of the presence of a cofactor. After isolation of the active material, spectral and degradative studies (131) indicated that the active factor was a derivative of adeninylcobamide cyanide, in which the cyanide group was replaced by an adenine nucleoside. In subsequent studies by Barker and his group (6) the corresponding coenzyme derivative of cyano-B_{12} was isolated (Fig. 1), although both the structure of the adenine nucleoside, characteristic of all the cobamide coenzymes, and its linkage to the cobalt were still not known at that time. The case of crystallization of the coenzyme derivative of cyano-B_{12} made it possible for Lenhert and Hodgkin (67) to carry out X-ray crystallographic investigations. These elegant studies elucidated the exact structure of the coenzyme. As shown in Fig. 1, the coenzyme differs from the vitamin only in that, in place of cyanide, there is a 5′-deoxyadenosyl group attached to cobalt by the C^5 of the 5′-deoxyribose moiety. The cobalt, in the coenzyme and the vitamin, is in the trivalent state. The structure of other related cobamides mentioned in this review are explained in the legend of Fig. 1. The coenzyme was found to be the major cobamide derivative detected in a variety of microorganisms (126) as well as

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Folate. Washed, packed platelets were suspended, in a 1 per cent V/V concentration, in 0.05 M phosphate buffer, pH 6.2, containing 0.5 per cent ascorbate. The suspensions were sonicated, centrifuged and the supernatants stored as described for B_{12} assay. Folate

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in animal liver (124, 130) accounting for as much as 80% of the cobamide compounds present. The lability of the carbon-to-cobalt bond in deoxyadenosyl-B_{12} (see below) very likely accounts for the failure to isolate the coenzyme previously.

CHEMISTRY

The presence of a cobalt-to-carbon bond in the B_{12} coenzymes (Fig. 1) accounts for many of the unique properties of these compounds. Visible light, cyanide, and acid (131) cleave the cobalt-carbon bond.

Light Inactivation

Brady and Barker (15) showed that, in an anaerobic environment, visible light causes a homolytic cleavage of the carbon-to-cobalt bond in the coenzyme, yielding B_{12r}, a one-electron reduced derivative of cyano-B_{12} containing divalent cobalt. Upon the addition of air, oxidation of B_{12r} to hydroxy-B_{12} occurs. Two adenine-containing compounds, first separated by chromatography on a Dowex-50 resin (131), are formed during light degradation of deoxyadenosyl-B_{12} in the presence of oxygen. These compounds have been identified as adenosine-5-aldehyde (51, 53) and 8:5′-cyclic-adenosine (52) (Fig. 2).

Acid Hydrolysis

Treatment with 1 N HCl at 100 C for 20 min cleaves both the cobalt-carbon bond in the coenzyme as well as the nucleoside bond and liberates free adenine

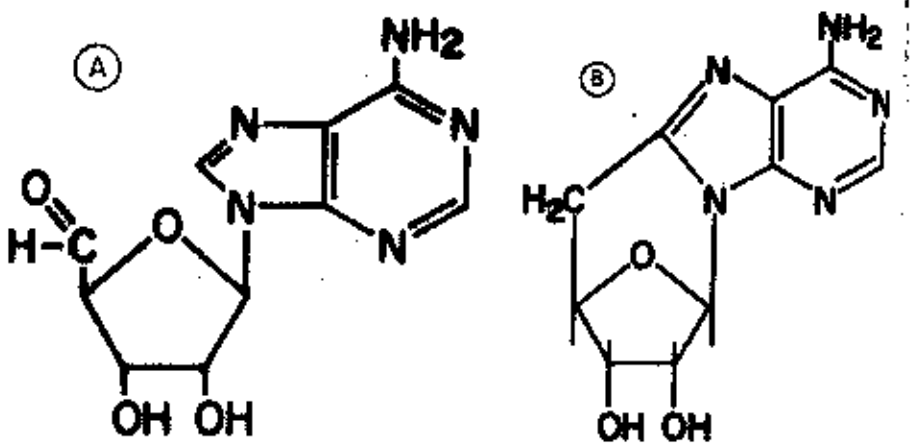
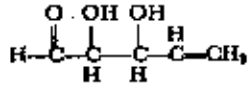


FIG. 2. Structures of adenine-containing compounds formed by light inactivation of the cobamide coenzyme *A:* adenosine-5-aldehyde and *B:* 8:5′-cyclic-adenosine.

and a sugar derivative (131). Hogenkamp and Barker have identified the sugar produced after acid hydrolysis as 2,3-dihydroxy-Δ 4-pentenal (50).



Cyanide

Alkaline cyanide readily cleaves the carbon-to-cobalt bond in deoxyadenosyl-B_{12}, forming cyano-B_{12}. The deoxyadenosyl nucleoside also is cleaved, liberating free adenine and the cyanohydrin of 2,3-dihydroxy-Δ-4-pentenal (53).

Chemical Synthesis of Deoxyadenosyl-B_{12} and Related Derivatives

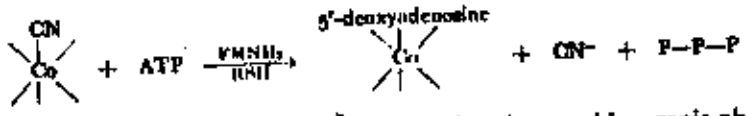
A notable advance in the understanding of the chemistry of deoxyadenosyl-B_{12} has come from studies by Smith et al. (106) on the chemical synthesis of deoxyadenosyl-B_{12} and its derivatives, i.e., cobamide compounds containing a carbon-to-cobalt bond. The general technique involves a two-electron reduction of hydroxy-B_{12} or cyano-B_{12} to a derivative of the vitamin containing univalent cobalt (hydrido-B_{12}). The reduction is readily performed with $NaBH_4$, chromous acetate, or zinc dust. Hydrido-B_{12} has characteristics of a metal hydride (120). It is stable only under anaerobic conditions but rapidly reacts with alkylating agents to form the corresponding alkyl-B_{12} derivative. Thus, on reacting hydrido-B_{12} with 5′-tosyl-2′3′-isopropylidene adenosine, a derivative was obtained which could be readily converted to deoxyadenosyl-B_{12} (B_{12} coenzyme) (106). In analogous fashion methyl-B_{12}, ethyl-B_{12}, etc., could be prepared by reacting hydrido-B_{12} with the appropriate alkyl halide. Tackett et al. (120) have shown that hydrido-B_{12} is oxidized to B_{12r} under anaerobic conditions, the rate of the reaction being dependent on the pH of the medium.

Dolphin et al. (27) have shown that alkyl-B_{12} compounds can be prepared by the reaction of an alkyl halide with sulfito-B_{12}, the latter compound prepared by reaction of hydroxy-B_{12} with a sodium hydrosulfite. It is postulated in this case that the active cobamide species, reacting with the alkyl halide, contains cobalt in the divalent state.

Enzymatic Synthesis of Deoxyadenosyl-B_{12}

Cell-free systems that can convert cyano-B_{12} or hydroxy-B_{12} to deoxyadenosyl-B_{12} have been described from *Propionibacterium shermanii* (16, 17) and *Cl. tetanomorphum* (88, 89, 132, 133). The reaction requires ATP, reduced flavin, and a sulfhydryl compound. ATP serves as a substrate, transferring a 5′-deoxyadenosyl moiety intact (86) to the vitamin to form the coenzyme. The three phosphates of

ATP in the *Cl. tetanomorphum* system are released in the form of inorganic tripolyphosphate (89), and thus the reaction is described as follows:



In the enzymatic system from *P. shermanii*, pyrophosphate and inorganic phosphate are the products of the reaction (17).

The need for reduced flavin suggests that the reaction may involve an initial reduction of the vitamin to a more reduced cobalt derivative, which could condense with ATP to form the coenzyme. This reaction would be analogous to the chemical synthesis of deoxyadenosyl-B_{12} described by Smith et al. (106). However, studies on the release of cyanide from the vitamin and spectral changes during coenzyme synthesis (193) have indicated that a reduced form of the vitamin is not an obligatory intermediate during coenzyme synthesis. It should be noted that a variety of cobamide compounds serve as substrate in the enzymatic reaction, including factor B (17). In fact, the deoxyadenosyl derivative of factor B has been isolated from natural sources (85), which suggests that the carbon-to-cobalt bond may be formed at an early stage (factor B or before) in the biosynthesis of the B_{12} coenzyme.

Role of Cyano-B_{12} in Enzymatic Reactions

There are now four defined enzymatic reactions which show a requirement for a cobamide compound. Deoxyadenosyl-B_{12} has been shown to be required in three of these reactions, and a cobamide-containing protein is involved in methionine formation.

Shortly after the demonstration that a cobamide coenzyme was essential for the isomerization of glutamate to β-methylaspartate (3), the isomerization of methylmalonyl CoA to succinyl CoA was also shown to be a cobamide coenzyme-dependent reaction (41, 66, 107, 112, 115, 116). Abeles and Lee (1) then demonstrated that the enzymatic conversion of ethylene glycol to acetaldehyde (or propylene glycol to propionaldehyde), catalyzed by extracts of *Aerobacter aerogenes*, required a cobamide coenzyme. The reactions requiring deoxyadenosyl-B_{12} are summarized in Fig. 3. Although the glycol dehydrase reaction is quite different from the two isomerase reactions, all three reactions can be pictured as involving a hydrogen transfer during the course of the reaction. In methionine synthesis a cobamide-containing protein (39, 59, 121) functions in the transfer of the methyl group from N^5-methyltetrahydrofolate (methyl-folate-H_4) (62–64, 101, 134) to homocysteine. S-adenosylmethionine (AMe) (73, 100) and a reducing system (42, 63) are also needed for this reaction. Recent studies on these four reactions are discussed below.

Glutamate isomerase. Earlier tracer studies demonstrated that carbon atom 3 of glutamate becomes the branched methyl of β-methylaspartate (81). The reaction

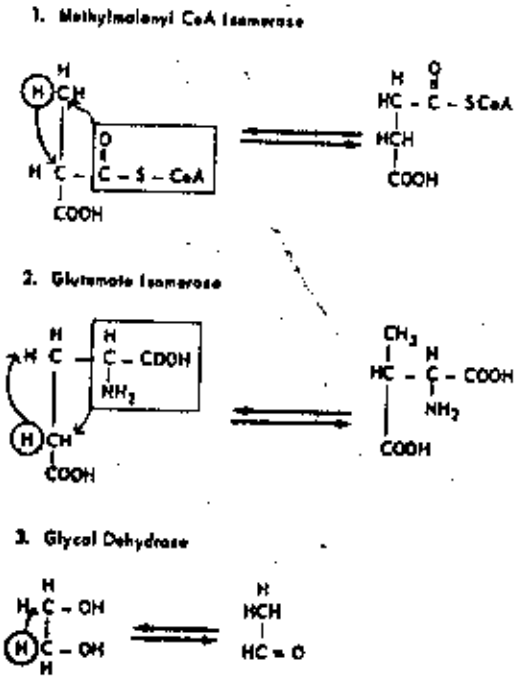
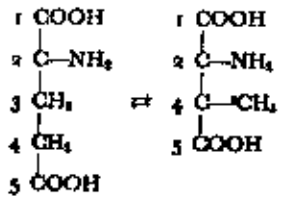


FIG. 3. Enzymatic reactions requiring a cobamide coenzyme.

can be thought to involve a transfer of the glycine moiety (carbons 1 and 2) of glutamate to carbon 4 of glutamate, to yield β-methylaspartate labeled as follows:



Two enzymes were thought to be involved in this reaction, although recent studies by Suzuki et al. (118) have shown that only one protein fraction is needed when the reaction is performed under anaerobic conditions. The manner in which the second protein stabilizes the isomerase in air has not been explained. α-Ketoglutarate and free ammonium ion have been eliminated as intermediates in the reaction (118). In order to determine whether the third hydrogen of the branched methyl group of β-methylaspartate comes from glutamate (presumably from the C-4 methylene group) or from the solvent, Iodice and Barker (54) carried out the reaction using either tritiated or deuterated water. The results showed that the third hydrogen atom of the branched methyl group formed in the isomerization of glutamate is

derived from the substrate and not the solvent. It is shown below that similar results have been obtained with methylmalonyl CoA isomerase and diol dehydrase. In both cases there is no incorporation of solvent hydrogen during the course of the reaction.

Sprecher and Sprinson (111) have investigated the stereospecificity of the glutamate isomerase reaction using β-methylaspartate labeled with deuterium in the β-position. They could distinguish, by degradation of the resulting glutamate formed, whether the hydrogen atom that replaces the leaving glycine moiety results in a retention or inversion of configuration at the β-carbon. The results showed that an inversion occurs, and the authors speculate that the cobamide-dependent rearrangement may proceed by a carbonium ion mechanism.

Glycol dehydrase. This reaction has been studied with a purified enzyme from *Aerobacter aerogenes* (1). Although glycerol is not a substrate of this enzyme, a similar cobamide-dependent enzyme catalyzing the conversion of glycerol → β-OH propionaldehyde has been demonstrated by Smiley and Sobolov (104) with extracts of *Lactobacillus* 208-A. In the glycol dehydrase reaction, as in the other reactions requiring the coenzyme form of the vitamin, a hydrogen transfer occurs. Studies by Brownstein and Abeles (19) with deuterated and tritiated water showed no incorporation of solvent hydrogen into the reactants. Incubation of the purified enzyme from *Aerobacter aerogenes* with coenzyme yielded inactivation of the enzyme as well as inactivation of the coenzyme (65). The inactivation of deoxyadenosyl-B_{12} was associated with spectral changes, a suggestion that it was decomposed to hydroxy-B_{12}. Since hydroxy-B_{12} and cyano-B_{12} inhibit the enzyme, the breakdown of deoxyadenosyl-B_{12} to hydroxy-B_{12} could account for the observed loss of enzyme activity. An excellent correlation was observed between spectral changes, due to decomposition of deoxyadenosyl-B_{12}, and enzymatic inhibition. The ability of the enzyme to cleave the carbon-to-cobalt bond in deoxyadenosyl-B_{12} may be a common feature of the mechanism of action of deoxyadenosyl-B_{12} in this and other cobamide coenzyme-dependent reactions. Evidence has also been obtained, from studies on the inhibition of the reaction by a sulfhydryl inhibitor, *p*-chloromercuribenzoate, that the binding of deoxyadenosyl-B_{12} to the enzyme involves a sulfhydryl group on the enzyme.

Methylmalonyl CoA isomerase. This reaction has been demonstrated in both microorganisms and mammalian tissues and is an important reaction in the metabolism of propionate (propionyl CoA → methylmalonyl CoA $\xrightarrow{B_{12}\text{ coenzyme}}$ succinyl CoA). Although the B_{12} coenzyme could readily be separated from the enzyme in bacterial extracts, resolution of the animal holoenzyme was achieved only after acid ammonium sulfate treatment (66). The mammalian enzyme, unlike the bacterial enzyme, is active only with the coenzyme form of the vitamin (containing 5,6-dimethylbenzimidazole in the nucleotide portion), not with the coenzyme form of adeninylcobamide cyanide (see Fig. 1). The work of Lynen and his group (32) showed that 2-C^{14}-methylmalonyl CoA was isomerized to 3-C^{14}-succinyl CoA during the course of the reaction, a demonstration that the thioester carboxyl group (see Fig. 3), and not the free carboxyl group, migrates. They have also established that no solvent hydrogen is incorporated into the reactants during

the isomerization reaction (86). Experiments with methylmalonyl CoA double-labeled with C^{14} have proved that the reaction involves an intramolecular, not intermolecular, group transfer (57, 90).

Methionine formation. Helleiner and Woods (45) first demonstrated a role of cyano-B_{12} in a cell-free system capable of synthesizing methionine. Subsequent work from several laboratories (39, 42, 59, 62–64, 73, 100, 101, 121, 134) has helped to elucidate the complex reactions by which the hydroxymethyl group of serine becomes the methyl group of methionine, as shown below:

a) serine + folate-H_4 → 5,10-methylene-folate-H_4 + glycine

b) 5,10-methylene-folate-H_4 $\xrightarrow{FADH_2}$ 5-methyl-folate-H_4

c) 5-methyl-folate-H_4 + homocysteine $\xrightarrow[\substack{B_{12}\text{-protein}\\ \text{reducing system}}]{AMe}$ methionine + folate-H_4

Reaction c is of special importance since it permits, at the enzyme level, an explanation for the known interrelationships among cyano-B_{12}, folic acid, and 1-carbon metabolism. Prefolic A, a reduced folate compound of unknown structure, was isolated by Donaldson and Keresztesy (28) in 1959 from animal liver where it constitutes a major fraction of the folate compounds (83). Subsequently Larrabee et al. (62, 63) reported the isolation of a methyl-containing folate derivative, 5-methyltetrahydrofolate, formed by bacterial enzymes as an intermediate in methionine biosynthesis from 5,10-methylenetetrahydrofolate. The identification of this new intermediate by the latter investigators permitted the ordering of the enzymatic reactions essentially as shown in *reactions b* and *c*, except for the finding of Mangum and Scrimgeour (73) that adenosylmethionine could replace ATP originally included in the scheme for *reaction c*. From the distinctive properties of 5-methyltetrahydrofolate and prefolic A as a growth factor for microorganisms (both support growth of *L. casei* but not *S. faecalis* and *L. citrovorum*) and from the absorption spectra of the two compounds it was realized that they were identical (29, 63). 5-Methyltetrahydrofolate was subsequently synthesized chemically by Keresztesy and Donaldson (58) and by Sakami and Ukstins (101) and has been shown by the latter investigators to be also a precursor of methionine in the reaction catalyzed by extracts of pig liver.

Thus, the transfer of the methyl group from methyl-folate-H_4 to homocysteine appears to be the final reaction in methionine formation in both bacterial and animal systems. The need for a cobamide protein for this reaction in *E. coli* extracts has stimulated interest in this methyl transfer, although it is apparent from the work of Woods and co-workers (38) that a second system, not cobamide-dependent, exists in many microorganisms which synthesize methionine. Triglutamyl rather than monoglutamyl derivatives of folate-H_4 are the intermediates in this pathway. This alternate reaction requires neither a cobamide protein nor the other complex requirements of the cobamide-dependent system. As is discussed below, however, the evidence indicates that the animal system catalyzing methionine formation is cobamide-dependent.

A notable advance in understanding the role of the vitamin in this reaction

resulted from studies with methyl-B_{12}, an alkyl analogue of deoxyadenosyl-B_{12} (see Fig. 1). This cobamide derivative, synthesized by Smith et al. (106), was tested in a methionine system of *E. coli* by Guest et al. (40). Not only could methyl-B_{12} form the holoenzyme, but more important this cobamide derivative enzymatically transferred its methyl group to homocysteine. Studies by Weissbach et al. (135) have shown that ethyl-B_{12} and β-propionate-B_{12} also can form an active cobamide protein, although other alkyl-B_{12} analogues inhibit the reaction. The finding that B_{12r} also satisfies the cobamide requirement (134) led to the speculation that the active species might be a reduced cobamide prosthetic group. This could accept a methyl carbonium ion from methyl-folate-H_4 and subsequently transfer it to homocysteine with the regeneration of the active reduced cobamide bound to protein (Fig. 4).

It appears that cyano-B_{12} must be reduced in order to be active in the enzymatic reaction. A reducing system is also needed to maintain the cobamide protein in the reduced state. Although the mechanism by which deoxyadenosyl-B_{12} functions in the isomerase and dehydrase reactions (Fig. 3) is not known, the possibility that a reduced cobamide on the enzyme is the active form in these reactions should be considered. The data show that AMe functions catalytically in the methionine-forming reaction (73), presumably in the initiation of the transfer of the methyl group from methyl-folate-H_4 to the cobamide protein, although its mode of action is still unknown. With catalytic amounts of enzyme, the methyl group of AMe is not transferred to homocysteine, although a very slow enzymatic transfer of the methyl group from AMe to hydroxy-B_{12} to form methyl-B_{12} has been reported by Foster et al. (33). This group has also postulated that a reduced cobamide-containing protein functions in the methionine synthesizing system.

Less is known about methionine synthesis in animal extracts. Although animal nutritional studies have implicated cyano-B_{12} in this reaction, there is only indirect evidence thus far that the reaction is similar to the cobamide-dependent reaction studied in microorganisms. The methionine-forming system from liver requires AMe and a reducing system, similar to the cyano-B_{12}-dependent reaction in *E. coli*. Although a definite cobamide dependency for the reaction has not been obtained, partial purification of the enzyme from both hog and chicken liver has provided evidence that the vitamin (or a derivative) is attached to the enzyme (20, 25). It has also been shown that partially purified fractions from animal liver can transfer the methyl group of methyl-B_{12} to homocysteine (134). Nutritional studies indicated

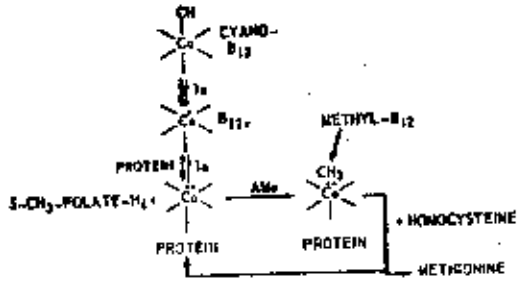


FIG. 4. Proposed scheme for the transfer of the methyl group from methylfolate-H_4 to homocysteine [taken from Weissbach et al. (44)].

that cyano-B_{12}-deficient chicks have very low levels of methyl-folate-H_4-homocysteine and methyl-B_{12} homocysteine transferase activities (25), the levels of which can be restored by administration of the vitamin for 24–48 hr prior to sacrificing the animals. All the above evidence suggests that the system from animal liver is quite similar to the cobamide-dependent system seen in *E. coli*.

The ability of methyl-B_{12} to function in methionine formation has stimulated interest in this derivative with respect to its function as a methyl transferring agent in other systems. Studies on methane formation with bacterial systems have shown an energy-dependent enzymatic conversion of the methyl group of methyl-B_{12} to methane (14, 139). Poston et al. (91) have also described a system in which the methyl carbon of acetate is derived from methyl-B_{12}.

Other Metabolic Functions of Vitamin B_{12}

A possible role of cyano-B_{12} in deoxyribose formation has come from studies with *Lactobacillus leichmannii* (11, 30, 74, 103, 108). This organism requires cyano-B_{12} for growth although this requirement can be replaced by any one of a number of deoxynucleosides. The available evidence indicates that the vitamin functions in the reductive step in which a ribonucleotide is converted to a deoxyribonucleotide. This has not been shown on the enzymatic level as yet. Although the glycol dehydrase reaction, studied by Abeles (1), is analogous to the conversion of ribose to deoxyribose, ribose and its derivatives are not substrates. Reichard (95) has obtained a cell-free system from *E. coli* that can convert a number of ribonucleotide diphosphates to their corresponding deoxynucleotides. Although the enzyme system has been purified, and appears also to involve a heat-stable protein (80), no evidence for a role of cyano-B_{12} or deoxyadenosyl-B_{12} has been reported. A similar system from animal tissues has so far failed to implicate cyano-B_{12} in deoxyribose formation (79). It will be of great interest to see whether the vitamin functions directly in deoxyribose formation and thus explain the results with *L. leichmannii*, or indirectly in an hitherto unknown manner. It has been postulated that there is abnormal formation of DNA in pernicious anemia (9) (see below), and it may be that the metabolic alterations in *L. leichmannii* and pernicious anemia are closely related.

Although a relationship exists between the cyano-B_{12} and the sulfhydryl content in animal tissues (31, 55, 56, 69, 70, 94), the importance of these earlier observations has not been assessed. Recently, attention has again focused on this aspect of cyano-B_{12} chemistry, with the finding that the cobamide dependency in the CO_2-pyruvate exchange reaction (93) is observed only under aerobic conditions (87) and is related to the ability of various cobamide derivatives to catalyze the oxidation of monothiols present in the incubation (87). How this cobamide-sulfhydryl interaction stabilizes the enzyme is not known. Since this interaction utilizes the oxygen present in the incubation, it may be a matter of making the incubations anaerobic, or the observed oxidation of the sulfhydryl compounds could be associated with a reduction of essential SH groups on the enzyme. A similar aerobic effect of cyano-B_{12} and other cobamides has also been observed in methionine synthesis with liver

extracts (20, 134). It is also of interest that the cyano-B_{12} requirement for *Lactobacillus lactis* Dorner is not seen if the organism is grown anaerobically or in the presence of reducing substances (61). This observation may represent an in vivo example of the role of cobamide compounds in the protection of oxygen-sensitive enzyme systems. It would not be surprising if this function of the vitamin plays an important role in animal nutrition.

A cobamide coenzyme requirement has also been reported for the fermentation of lysine to acetate and butyrate catalyzed by extracts of *Clostridium* M-E (113). However, the reactions in which the coenzyme functions in this system are not known.

Role of Cyano-B_{12} in Animal Metabolism

As our knowledge of the various enzymatic functions of cyano-B_{12} has increased, it has become possible to obtain a clearer picture of the important reactions involving the vitamin in animal tissues. We described in the previous sections three enzymatic reactions involving the coenzyme form of the vitamin, as well as the role of a cobamide-containing protein in methionine synthesis. In addition there are several reactions in which the vitamin or derivatives have been implicated, although unequivocal in vitro data are lacking. These reactions, described earlier in this review, are summarized in Table 1.

In only one of these, the methylmalonyl CoA isomerase reaction, has a cobamide participation at the enzymatic level been demonstrated in animal tissues. There is convincing evidence that a cobamide protein functions in methionine synthesis in animal tissue, and indirect evidence has accrued to suggest a similar participation in deoxyribonucleotide formation and sulfhydryl metabolism.

Propionate Metabolism. Marston et al. (75) suggested that the metabolic block in cobalt deficiency, observed in sheep, involves the utilization of propionic acid. Subsequently, in animals on cyano-B_{12}-deficient diets other workers observed a decrease in the conversion of methylmalonyl CoA to succinyl CoA and restoration of this depressed activity with in vivo administration of cyano-B_{12} (41, 107). The sheep enzyme, methylmalonyl CoA isomerase, has been extensively purified and resolved into an apoenzyme and a prosthetic group, deoxyadenosyl-B_{12} (66). These studies are the only demonstration in animal tissues in which an enzyme has been purified and resolved into an apoenzyme and a cobamide prosthetic group. Of

TABLE 1. *Summary of reactions involving cobamide compounds*

Reaction	Active Cobamide	Occurrence
Glutamate isomerase	Coenzyme	Bacteria
Methylmalonyl CoA isomerase	Coenzyme	Bacteria and animals
Glycol dehydrase	Coenzyme	Bacteria
Methionine formation	Cobamide protein	Bacteria and animals
Lysine fermentation	Coenzyme	Bacteria
DNA formation (uncertain)	Unknown	Bacteria and possibly animals
Sulfhydryl reduction	Unknown	Bacteria and animals

additional interest are the observations that an increased excretion of methylmalonic acid occurs in individuals with pernicious anemia (7, 23). The increased excretion, which was observed in cyano-B_{12}-deficient rats, was decreased by the presence of small amounts of cyano-B_{12} in the diet, but not by administration of folic acid, vitamin F_1, or selenium (8).

Cyano-B_{12}-folate interrelationship. Ever since the observation that the *Lactobacillus casei* growth factor or folic acid led to a reticulocytosis and improvement of the anemia in patients with pernicious anemia (109), the interrelationship between folate and cyano-B_{12} has been studied intensively. It was soon observed that the clinical remissions following folic acid therapy were temporary and partial in that they did not correct the neurological signs of pernicious anemia (43, 44, 125). Dawborn et al. (24) found that cyano-B_{12}-deficient sheep, grazing on cobalt-deficient lands, had markedly reduced concentrations of liver folic and folinic acid. Nutritional studies also indicated that signs of folic acid deficiency in fowl could be accentuated by cyano-B_{12} administration (82).

A divergent, but soon to be related, nutritional equivalence was observed between cyano-B_{12} and methyl group donors, i.e., methionine. Newborn chicks rendered deficient in cyano-B_{12} were found to grow poorly unless the vitamin was restored (12, 72, 117). However, methionine, and to a lesser extent choline, could alleviate the growth impairment and exert a sparing action on the vitamin requirement (35). Although there was a definite growth impairment, cyano-B_{12}-deficient animals did not have signs of a macrocytic acemia (26). Fox and her co-workers (34) concluded that in order to demonstrate a nutritional deficiency of cyano-B_{12} it was necessary to reduce the methionine content of the diet to levels suboptimal for growth. Despite the inhibition of growth response on cyano-B_{12}-deficient diets, there was no evidence of a complete absence of cyano-B_{12} in the livers of deficient animals.

Further data showing a folate-cyano-B_{12} interrelationship have come from studies on histidine metabolism in animal tissues (76, 119). It was shown that the formimino group of formimino-glutamic acid (FIGLU) is transferred to folate-H_4 to synthesize 5-formimino-folate-H_4. This transfer is similar to the previously described transfer of the formimino group of formimino-glycine to folate-H_4 in *Clostridium cylindrosporum* (92). The urinary excretion of FIGLU after a histidine load was found to be increased in patients with folic acid deficiency, as well as those receiving folic acid analogues (18, 71). Thus, FIGLU excretion appeared to be a sensitive index of the intracellular availability of folate-H_4 in man. Similar increases in urinary FIGLU excretion after histidine loading were observed in rats (102) and chicks (36) which were on either folic acid, or perhaps of more importance, on cyano-B_{12}-deficient diets. Also, increased FIGLU excretion was observed in some, but not all, patients with pernicious anemia (60, 141).

The metabolic basis of the alterations in FIGLU excretion, as well as the nutritional interrelationships among cyano-B_{12}, folic acid derivatives, and methionine, can be understood more clearly in light of in vitro studies on the biogenesis of the methionine methyl group. From the in vitro studies presented earlier in this review, it is apparent that the terminal reaction in the biosynthesis of methionine

involves a cobamide-containing protein. Thus, in cyano-B_{12} deficiency there would be a block in the conversion of 5-methyl-folate-H_4 to methionine and a resulting failure to regenerate folate-H_4. This inability to regenerate folate-H_4 and other "active" folates (10-formyl, 5,10-methenyl and 5,10-methylene-folate-H_4) would yield an alteration in purine and thymidine methyl synthesis as well as affect the catabolism of histidine. These reactions and interconversions are shown in Fig. 5. Thus, one important result that would be expected from cyano-B_{12} deficiency is an alteration in the partition of folate compounds in the body due to the inability to transfer the methyl group from 5-methyl-folate-H_4 to homocysteine. Indeed, there is now ample in vivo evidence in both animals and man that the folate partition is affected by the cyano-B_{12} and methionine content in the diet. In chicken and rat liver, 5-methyltetrahydrofolic monoglutamate was determined to be the folate derivative in greatest concentration (83, 84). Noronha and Silverman (84) observed that an increase in dietary methionine in control or cyano-B_{12}-deficient rats caused an alteration in liver folate distribution. The 5-formyl-, 10-formyl-, and unsubstituted folate-H_4 concentrations were increased while that of methyl-folate-H_4 was decreased. Cyano-B_{12} replenishment in deficient rats was observed to cause an alteration similar to that induced by dietary methionine but to a lesser extent. Noronha and Silverman (84) were the first to suggest that methionine or cyano-B_{12} could function by influencing the release of functional forms from stores of folate trapped as methylated folates. The pronounced effect of methionine to reverse the

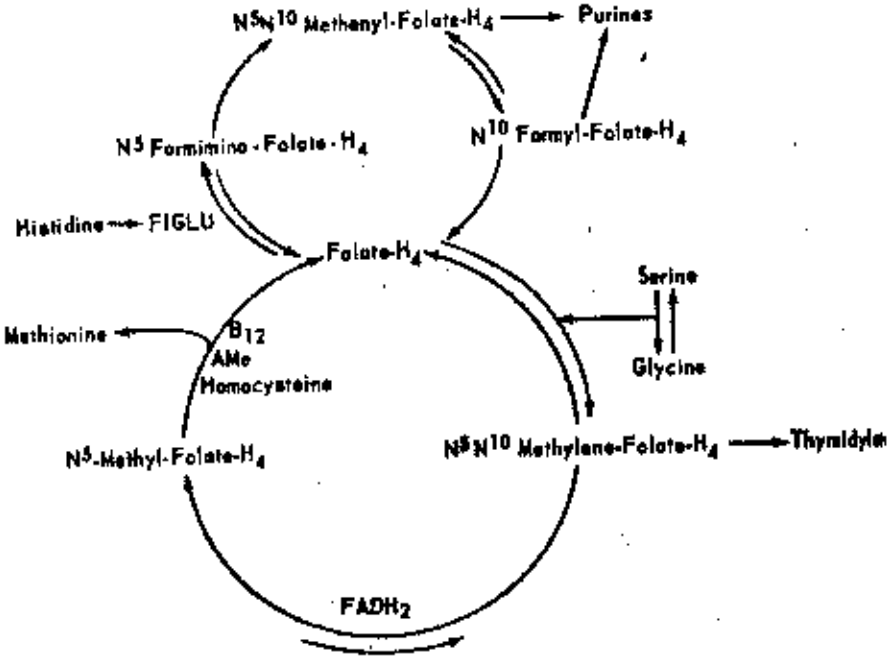


FIG. 5. Folate cycle in animal metabolism—a simplified scheme showing some of the important interrelationships involving folate metabolism.

folate pattern in both normal and cyano-B_{12}-deficient animals was also seen in patients with pernicious anemia (46). Elevated urinary FIGLU excretion is suppressed by dietary administration of methionine. The exact mechanism of the methionine effect on the folate partition is still unknown.

The significance of the folate partition in normal subjects as well as patients with pernicious anemia was not apparent until the identification of most of the serum *Lactobacillus casei* growth factor as 5-methyl-folate-H_4 monoglutamate by Herbert, Larrabee, and Buchanan (47). Determinations of folic acid activity in pernicious anemia patients indicated that while the plasma concentrations of *Streptococcus faecalis* growth factors (principally formylated and unsubstituted forms of folate-H_4) were reduced in approximately 50% of the patients (21), the mean serum concentrations of *L. casei* factor were higher than in normals (48, 128). In addition to the cyano-B_{12} deficiency, the level of *L. casei* factor was found by Mollin et al. to be correlated with the severity of the anemia in patients with pernicious anemia (128). The highest concentrations of serum *L. casei* factor were found in the least anemic patients, suggesting that in the presence of an adequate folic acid intake one may not see the hematological lesion. This may explain the absence of anemia in cyano-B_{12}-deficient animals when their folic acid intake is sufficient. These findings were interpreted as indicative of a decreased utilization of the *L. casei* factor (principally 5-methyl-folate-H_4) in cyano-B_{12} deficiency. Herbert and Zalusky (48) found that cyano-B_{12} therapy led to a reduction in the serum *L. casei* factor. Results consistent with an underutilization of serum *L. casei* factor were obtained in studies of serum *L. casei* factor disappearance as well as tritiated folate disappearance in individuals with pernicious anemia (22, 48, 78).

Deoxyribonucleotide formation. Although an imbalance of folate compounds would yield an altered nucleic acid metabolism, an alternative hypothesis for possible derangement in nucleic acid metabolism in cyano-B_{12} deficiency has focused on the reduction of ribonucleotides to deoxyribonucleotides. Microspectrophotometric analyses of the megaloblastic cells in pernicious anemia marrow indicate that the cellular content of DNA is normal or elevated and that of RNA is elevated (136). Other workers using the same techniques, however, have found no difference in the nuclear content of deoxyribose in megaloblasts or normoblasts (96). Glazer et al. (37) measured the RNA/DNA and uracil/thymine ratio of marrow cells from control subjects and patients with megaloblastic anemia. In the marrow cells from anemic patients, both the RNA/DNA and uracil/thymine ratios were elevated compared to the controls. Both ratios decreased significantly after therapy and prior to the observed reticulocytosis.

Additional studies have focused on the incorporation of nucleic acid precursors into megaloblastic and normoblastic marrow cells. A prime difficulty of these studies is the heterogeneity of the marrow cell population for, even in severe megaloblastic anemia, megaloblasts account for up to only 70% of the total cell population (95). Thymidine and deoxyuridine were incorporated equally well into marrow cell DNA from controls or individuals with megaloblastic anemias due to either cyano-B_{12} or folic acid deficiency (68). There was no effect of folic acid, folinic acid, or cyano-B_{12} on these nucleoside incorporations. Thomas and Lochte (122) found

that labeled formate was not as readily incorporated into megaloblastic marrow DNA thymine as in the control population, but when incubated in the serum of pernicious anemia the incorporation increased in response to added cyano-B_{12}. Williams et al. (138) have studied the in vitro incorporation of adenine-8-C^{14} and cytidine-2-C^{14} into DNA and RNA bases of marrow from patients with megaloblastic or hemolytic anemia. In the megaloblastic marrow, an increased response to added cyano-B_{12} was observed in the DNA rather than the RNA bases. This effect was not observed in the normoblastic marrows in patients with hemolytic anemia.

Beck has postulated that the megaloblast is a result of a similar cellular alteration to that observed in either a thymineless *E. coli* mutant grown in the absence of thymine, or in *L. leichmannii* grown in limiting amounts of either cyano-B_{12} or any deoxynucleoside (9). The latter organism has a growth requirement which can be satisfied by either cyano-B_{12} or any of the four deoxynucleosides (103). Under the condition of limiting amounts of cyano-B_{12} or deoxynucleosides, a state of "unbalanced growth" is observed in which DNA synthesis is impaired while no alteration is observed in RNA or protein synthesis (10). As a result, the cellular RNA/DNA ratio is increased and the bacterial cells assume an elongated, filamentous shape. The "unbalanced growth" was reversed in *L. leichmannii* by increasing the concentration of either cyano-B_{12} or deoxynucleosides in the media. A similar effect of thymidine was not observed in tissue culture nor when thymidine was employed as a therapeutic agent in pernicious anemia (110). However, human marrow tissue apparently lacks a trans-N-deoxyribosidase which would catalyze the interconversion of the three other bases with the acceptor deoxyribose (98). No experiments have been reported as yet on the conversion of megaloblastic cells to normoblastic cells in tissue culture or on a therapeutic response with combinations of the four deoxynucleosides. Such a positive result would be anticipated if the deficiency of cyano-B_{12} is primarily a decrease in ribonucleotide reduction.

Sulfhydryl metabolism. Alterations in sulfur metabolism have also been reported in experimental cyano-B_{12} deficiency as well as in individuals with pernicious anemia. Ling and Chow (69) observed that blood glutathione levels were depressed in the deficiency state and that a restoration to normal values followed vitamin repletion. In contrast, Kasebakakar et al. (56), using iodinated casein to intensify the cyano-B_{12}-deficient state, observed a decrease in liver sulfhydryl and glutathione levels but elevated blood levels of these compounds. Stekol (114) failed to observe any significant difference in the incorporation of glycine or cystine into glutathione of cyano-B_{12}-deficient rats compared to control animals. A possible indication was obtained that the blood sulfhydryl alteration in cyano-B_{12} deficiency was due to an increase in the GSSG/GSH ratio of erythrocytes secondary to an effect on glutathione reductase (55). Additional observations indicative of a sulfhydryl defect were the observed increases in the urinary excretion of thiocyanate and taurine (123, 129, 140). The possible alterations in levels of intracellular glutathionine may be responsible for the in vivo alterations in glycolysis and lipid metabolism which have been observed (13, 70).

Finally, attention should be directed to the multiple levels of cyano-B_{12} action. Arnstein and White (2) have established that during growth on restricted cyano-

B_{12} intake the propionate metabolism is affected more severely than formate metabolism. However, cyano-B_{12} analogues that inhibit growth stimulate propionate metabolism whereas others that stimulate growth do not affect the metabolism of propionate in *Ochromonas malhamensis*.

In the cobalt deficiency disease of sheep, the defect in methylmalonyl CoA isomerization appears prior to the fatty infiltration of the liver, the latter presumably related to a decreased rate of methionine biosynthesis (75).

The inability of folic acid to reverse the central nervous system lesions of pernicious anemia represents yet another example where cyano-B_{12} participates in two separate metabolic pathways in animal tissue.

ADDENDUM

Blakley and Barker (*Biochem. Biophys. Res. Commun.* 16: 391, 1964) have recently obtained evidence of a cobamide requirement for deoxyribose synthesis in vitro. In these studies, using cell-free extracts of *Lactobacillus leichmannii*, the authors demonstrated the need for deoxyadenosyl-B_{12} in the conversion of cytidine monophosphate to deoxycytidine monophosphate.

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Decreased Serum B_{12} Levels With Oral Contraceptive Use

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Low serum vitamin B_{12} levels were found in healthy young women who were taking oral contraceptive agents (OCA). The fall in serum B_{12} values occurred rapidly and, in some instances, serum levels were indistinguishable from other forms of vitamin B_{12} deficiency. Serum B_{12}-binding proteins or tissue levels of vitamin B_{12} did not appear to be altered in association with the change in serum levels. Serum folic acid levels were also reduced in some women, but folate therapy had no effect on serum B_{12} levels. The mechanism by which oral contraceptives alter B_{12} metabolism is not clear and the pathophysiologic significance remains to be elucidated. Serum B_{12} values obtained in women taking OCA must be interpreted, however, in the light of this phenomenon.

A number of agents may affect vitamin B_{12} or folate metabolism.[1] Among these agents are the oral contraceptive drugs which appear to cause malabsorption of polyglutamic folate and may induce megaloblastic anemia in some women.[2-5] The infrequency of megaloblastic anemia secondary to the use of these agents appears obvious from the fact that millions of women used them for almost a decade before this untoward effect was recognized.

To our knowledge, oral contraceptive agents have not been reported to alter vitamin B_{12} levels. Bianchine et al[6] reported modest increases in the serum unsaturated B_{12}-binding capacity related to the use of these drugs; serum B_{12} levels in these subjects, however, were not mentioned.

The present report describes the occurrence of decreased serum B_{12} levels in women taking oral contraceptives and compares these women with pregnant subjects in whom low serum B_{12} levels also occur.[7-9]

Subjects

The study group comprised 20 healthy volunteers who had been taking oral contraceptive agents for periods of 2 to 60 months. All had normal dietary habits and none were taking other medication except for aspirin occasionally. The oral contraceptive agents in use at the time of the study were all of the combination type.

Twenty-six women in the last trimester of pregnancy were also studied. These women were all in good health and were eating normally. Most of them were receiving iron and vitamin supplements which contain 0.1 mg of folic acid and 2μg of cyanocobalamin. The vitamin B_{12} data for this group are included in our results because serum B_{12} values usually continued to decrease in pregnancy despite daily oral administration of this amount of the vitamin.[10]

Twenty-three women of comparable age who had neither been pregnant nor taken oral contraceptives for at least eight months served as controls. None were taking any medications except for aspirin occasionally.

Methods

Serum vitamin B_{12} was assayed by the radioisotope dilution technique of Lau et al[11] with the use of hemoglobin-coated charcoal. Unsaturated B_{12}-binding capacity (UBBC) was also measured with a radioisotope dilution technique as described by Gottlieb et al.[12] Separation of the alpha- and beta-binders was performed with the use of rapid diethyl aminoethyl (DEAE)-cellulose column chromatography as described by Retief et al.[13] In the latter two procedures, cyanocobalamin Co 57-B_{12} in a concentration of 2,200μμg/ml was utilized and unbound B_{12} was absorbed with hemoglobin-coated charcoal. All assays were performed within two weeks of obtaining the serum sample. When it was not possible to assay fresh sera, the samples were stored at −20 C (−4 F). Erythrocyte vitamin B_{12} level was assayed by the coated-charcoal technique of Kelly and Herbert.[14] Extraction and assay were performed on

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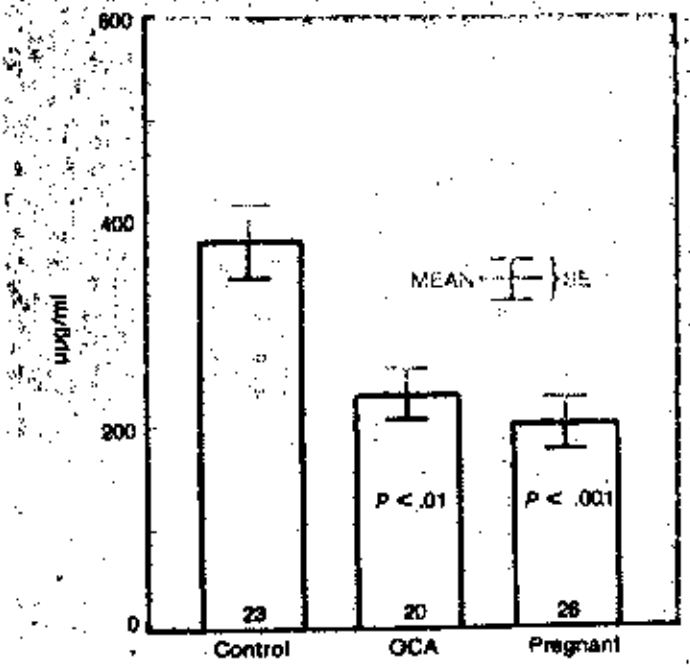


Fig 1.—Serum vitamin B_{12} levels for normal women, women taking oral contraceptive agents (OCA), and pregnant women. Number of subjects studied in each group is designated.

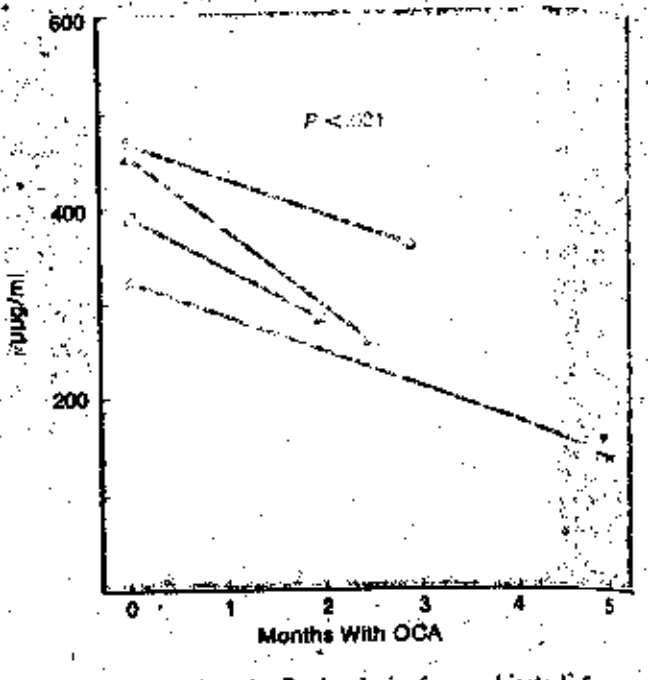
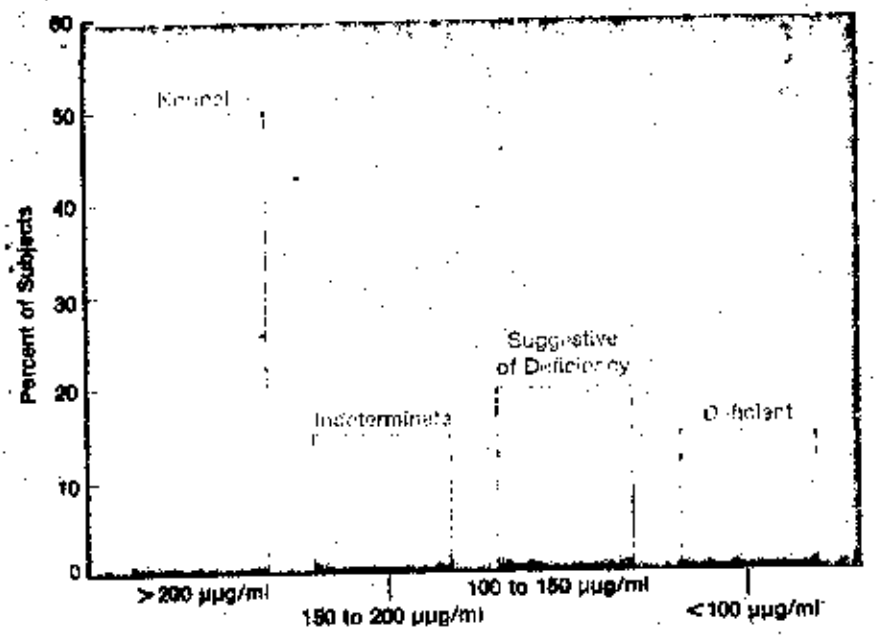


Fig 3.—Serum vitamin B_{12} levels in four subjects before and after taking OCA. The P value was determined by paired statistical analysis.

Fig 2.—Distribution of serum vitamin B_{12} values in 20 women taking OCA.



Mean Hematocrit Value and Neutrophil Lobe Averages*

	Control Mean (Range)	OCA Mean (Range)	Pregnant Mean (Range)
Hematocrit	40% (38-44)	41% (38-46)	35% (32-43)
Lobe No.	2.76	2.71	2.52
Average	(2.44-2.95)	(2.48-3.04)	(2.00-3.04)

*For normal women, women taking OCA, and pregnant women.

the day the blood was obtained. Serum folate level was assayed with *Lactobacillus casei* with the aseptic technique of Herbert.[13] Complete blood cell counts (CBC) and red blood cell (RBC) indices were determined by standard techniques. The average number of nuclear lobes in peripheral blood neutrophils was calculated for all subjects.[15]

Results

Serum Vitamin B_{12} Levels.—Women taking oral contraceptives were found to have a mean serum B_{12} level of 221µµg/ml which was 40% lower than the control mean of 372µµg/ml (Fig 1). This difference is statistically significant ($P<.01$). No statistical difference was found between women taking oral contraceptives and those in the last trimester of pregnancy. Although the mean serum B_{12} level of women taking oral contraceptives was within the normal range, 50% of these subjects had subnormal values (below 200µµg/ml) and 15% were in the clearly deficient range, below 100µµg/ml (Fig 2).

In five women taking oral contraceptives and in two normal subjects serum samples were collected two to three times each week

throughout one complete menstrual cycle and the serum B_{12} level was measured. There was no detectable difference in serum B_{12} levels related to the phase of the cycle.

Four women were studied prior to starting oral contraceptive use and again after two to five months of use (Fig 3). All four subjects had a reduction in serum B_{12} levels ranging from 34% to 58%. The differences were highly significant by paired *t*-test ($P < .001$). Neither UBBC nor serum folate values changed during this time.

B_{12}-Binding Proteins.—In contrast to pregnancy, the lower serum B_{12} levels found in women taking oral contraceptives were not associated with an increase in UBBC (Fig 4). As described by others[4,5] the UBBC was found to be significantly higher than normal ($P < .001$) in women during the last trimester of pregnancy. However, unlike Bianchine et al,[5] we could detect no elevation of the UBBC in women taking oral contraceptives. In addition, no differences in the elution characteristics of the binding proteins were seen. In each group, 70% to 90% of bound radioactivity was eluted with the fraction containing the beta-binder.

Tissue Vitamin B_{12} Stores.—Erythrocyte B_{12} values in women taking oral contraceptives ranged from 190μμg/ml to 430μμg/ml of packed RBCs with a mean of 312μμg/ml. Normal values in our laboratory range from 100μμg/ml to 500μμg/ml of packed RBCs.

As can be seen in the Table, no anemia or hypersegmentation was present in any of the subjects studied. Furthermore, RBC size was normal as determined on peripheral blood smear and by RBC indices.

Vitamin B_{12} Absorption.—Schilling tests, performed in two subjects taking oral contraceptives, showed normal results in both. One of these subjects had a 58% reduction in serum B_{12} level during five months of oral contraceptive use (Fig 3). The other subject had been taking oral contraceptives for two years and was found to have a serum level of 14μμg/ml.

Oral Contraceptive Effect on Serum Folate.—Measurement of serum folate in women taking oral contraceptives demonstrated that this vitamin was also significantly reduced when compared with normals (Fig 5). Normal values in our laboratory ranged from 4 to 30 ng/ml. Although the mean folate level found in women taking oral contraceptives was well within our normal range, six of 20 women had values below 4.0 ng/ml. Four of these six women also had subnormal serum B_{12} levels (<200μμg/ml), three of whom were below 150μμg/ml.

Result of Folate Therapy on Vitamin B_{12} Levels.—Two of the subjects with subnormal values for both serum folate and B_{12} were given folic acid (5 mg/day) orally while continuing to take oral contraceptives. In both subjects the serum folate rose to supranormal values (>30 ng/ml) but no change in the serum B_{12} levels occurred when remeasured one month after folate therapy was begun. One of these women was studied again six months after cessation of folic treatment and was again found to have subnormal values for both vitamins. The second subject had serum B_{12} and folate levels measured four months after cessation of folic acid therapy and was found to have a normal folate level, but the serum B_{12} was unchanged at a subnormal level.

A third woman with subnormal values for folate (3.9 ng/ml) and B_{12} (26μμg/ml) was given both folic acid (5 mg/day) and cyanocobalamin (1.0μg/day) orally for one month while continuing to take oral contra-

Fig 4.—Serum unsaturated B_{12}-binding capacity for normal women, women taking OCA, and pregnant women. Number of women studied in each group is designated.

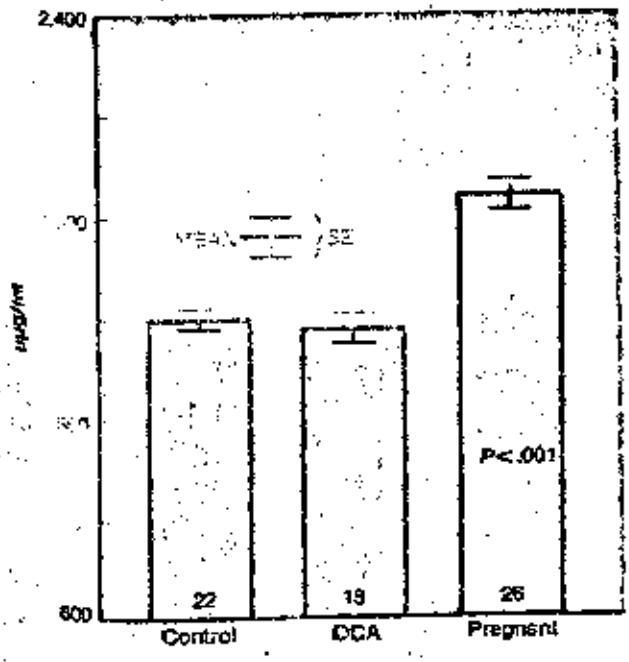


Fig 5.—Serum folic acid levels for normal women and women taking OCA. Number of women in each group is designated.

ceptives. Repeat assays at this time showed a supranormal serum folate level and a suboptimal elevation of serum B_{12} level to 153μμg/ml. Three months after discontinuing vitamin therapy, the serum folate level remained high normal (22 ng/ml) and the serum B_{12} value was unchanged in the subnormal range (170μμg/ml).

Comment

The data presented herein demonstrate a significant reduction of serum B_{12} levels in women taking oral contraceptives. This reduction can occur within five months and serum levels may fall to values indistinguishable from other forms of vitamin B_{12} deficiency. In spite of the drastic reduction in serum levels found in some women, no anemia or evidence of tissue depletion was detected. Also no detectable change in serum B_{12}-binding proteins occurred. This study also confirms the work of others in demonstrating a reduction in serum folate in women taking oral contraceptives. In addition, the simultaneous reduction of both serum B_{12} and folate levels was demonstrated in some of these women. Oral administration of folic acid had no effect on serum B_{12} values in three of these subjects, suggesting that the serum vitamin B_{12} decrease was not secondary to folate deficiency.

The mechanism by which serum B_{12} values are reduced in these women is not clear. Possibilities include the following: (*a*) malabsorption of B_{12}, (*b*) increased renal excretion, (*c*) factor(s) which interfere with the radioisotope dilution assay for this vitamin, or (*d*) enhanced tissue avidity.

Normal Schilling tests in two subjects and the rapidity with which serum levels were observed to fall make malabsorption unlikely. In addition, one subject demonstrated an increase in serum B_{12} level when a small oral dose was administered.

Renal excretion of vitamin B_{12} occurs primarily by glomerular filtration of the free vitamin.[17] For significant excretion to occur, serum B_{12} would either have to exceed the binding capacity of serum or be readily dissociated from the binding proteins. The normal UBBC and elution properties of binding proteins found in these women militate against this possibility.

At present we have no evidence of any factor(s) which might interfere with this assay. Supernatant controls measured with each sample tested demonstrated no significant residual binding in the sera which might result in a falsely low value. Dilution of serum from a subject taking oral contraceptives with serum from a normal subject resulted in values predicted by the dilution ratio. Thus, no evidence exists for a serum factor which might interfere with this assay.

Oral contraceptives might somehow enhance tissue avidity for B_{12} resulting in a redistribution of this vitamin. This could account for the normal RBC B_{12} values and the absence of anemia, macrocytosis, or granulocyte hypersegmentation in the face of low serum B_{12} levels. The fact that RBC levels were not greater than normal might be explained by the wide range of normal values or the differential avidity of tissues for B_{12}, or both.

Whether or not the low serum B_{12} levels found in women taking oral contraceptives has pathophysiologic significance remains to be determined. Prospective studies would be helpful in determining the extent of alterations in B_{12} metabolism which may occur with prolonged use of these agents. The possibility that megaloblastic anemia might result from the long-term use of these agents exists. The further possibility that neurologic damage due to indiscriminate use of folic acid in women taking oral contraceptives must be considered. Even if no pathophysiologic changes can be attributed to the reduced serum B_{12} levels found, interpretation of B_{12} results obtained in women taking oral contraceptives must be made with consideration of this phenomenon.

This investigation was supported in part by a grant from the National Vitamin Foundation.

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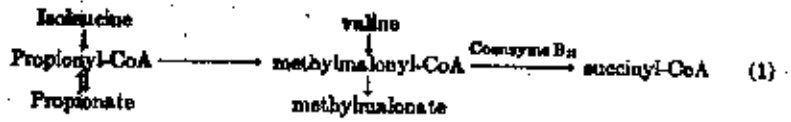
[215] Detecting Vitamin B_{12} Deficiency in Humans by Measuring Urinary Excretion of Methylmalonic Acid

By A. M. White

Vitamin B_{12} is a coenzyme for methylmalonyl-CoA isomerase (methylmalonyl-CoA CoA-carbonylmutase, EC 5.4.99.2). This means that humans who are deficient with respect to vitamin B_{12} cannot oxidize propionate through succinyl-CoA, Eq. (1), at an optimal rate. As a consequence they excrete amounts of methylmalonic acid that are in excess of normal.[1]



(1)

Loading tests, designed to increase even further the amounts of methylmalonate excreted by these subjects involve the administration of one of the major precursors of methylmalonate, namely, propionate, valine, or isoleucine. Of these precursors, valine gives the most reliable results.[1]

The normal daily excretion of methylmalonate cannot be stated with certainty until greater coverage has been made of the world population. Cox and White[1] considered 4.0 mg to be the largest amount excreted by hospital patients with serum B_{12} in the normal range and by normal controls. Gomperts,[2] however, has put the upper level of normal at 9.0 mg. This figure can rise to 500 mg in cases of severe deficiency disease, and when 10 g of valine is administered on the day of urine collection, up to 1.0 g of methylmalonic acid may be excreted.[2] Valine, however, does not affect the amount of methylmalonic acid excreted by normal controls or by patients with folic acid deficiency.

Gas chromatography is the method of choice for estimating the amount

[1] E. V. Cox and A. M. White, *Lancet* II, 853 (1962).
[2] D. Gomperts, J. Howel Jones, and J. F. Knowles, *Clin. Chim. Acta* 18, 197 (1967).

of methylmalonic acid in urine. This method allows a simultaneous check both of the purity and identity of the methylmalonic acid isolated whereas colorometric procedures,[3,4] although assuming prominence, cannot have this degree of certainty. While recoveries of methylmalonic acid from samples of urine can be high (>90%) it is considered advisable to add radioactively labeled methylmalonic acid to the urine for accurate work since recoveries may differ considerably from sample to sample.

Procedure

Reagents

Ether
Toluene
Valine
Na methylmalonate-3-^{14}C from New England Nuclear Enterprises
Sulfuric acid, 10 *N*
Acetic acid, 1 *N*
Diazomethane, freshly prepared from *N*-methyl-*N*-nitroso-toluene-*p*-sulfonamide
Octanoic acid
Anion-exchange resin (Dowex 1, 8% crosslinked, 200–400 mesh or equivalent) in acetate form

Collection of Urine. A 24-hour urine specimen is collected, covered with about 0.5 cm of toluene, and stored at 4° until required.

Preparation of Patients. About halfway through the 24-hour period, a 10 g load of pure valine dissolved in a little water and suitably flavored is given to the patient to drink.

Isolation and Estimation of Methylmalonic Acid. Precipitated solids in the 24-hour urine specimen are redissolved by gently warming and by adding, if necessary a little water. Sodium methylmalonate-3-^{14}C (5.2 μg, 0.01–0.05 μl) is then added and thoroughly mixed in by shaking. A 200-ml aliquot of the solution is brought to pH 1 with 10 *N* H_2SO_4, and the urine is extracted with ether (250–500 ml) overnight in a continuous extraction apparatus. The ether is removed by evaporation, and the extract is washed onto a column of anion-exchange resin (1.5 × 42 cm). The weak ether-soluble acids are eluted with acetic acid (800 ml, 1 *N*) and methylmalonic acid with hydrochloric acid (400 ml, 1 *N*). The total eluate from the column may be evaporated directly in a rotary evaporator, whereupon about 50% will be lost as a result of decarboxylation; or 50% may be returned to the

[3] A. J. Giorgio and G. W. E. Plaut, *J. Lab. Clin. Med.* **66**, 667 (1965).
[4] A. E. Green and G. D. Pegrum, *Brit. Med. J.* **3**, 591 (1968).

continuous ether extractor for overnight extraction, whereupon the recovery in this portion will be greater than 90%. If direct evaporation is used, the residue is dissolved in a little water (1.0 ml) and the aqueous solution is shaken with ether (20 ml). The ether solutions produced by one of these two procedures are evaporated to dryness. Octanoic acid (1.0–2.0 mg) or another acid of similar chain length is added as an internal standard for gas chromatography, and the acids are esterified with the minimum amount of diazomethane in ether in a graduated stoppered tube. At this point the amount of radioactivity is determined in a small aliquot (0.1 ml) to give the recovery of methylmalonic acid. Another suitable aliquot is directly injected into a gas chromatogram for quantitative estimation of the amount of methyl methylmalonate present.

Gas Chromatography. Examples of suitable stationary phases for use with an argon–ionization detector are 10% polyethylene glycol adipate or Apiezon L on Celite or Gas Chrom P. The instrument is programmed at 2°/minute from 78° to 123° at an operating voltage of 1200 V.

For a machine utilising a hydrogen flame ionization detector,[4] 10% polyethylene glycol adipate on 100–120-mesh Gas Chrom P has been used in a 5-foot long glass column, 4 mm internal diameter. At 87° the flow rates were: argon, 50 ml/min; hydrogen, 60 ml/min; air 600 ml/min. The amplifier attenuation was 50×10^{-11} A.

[4] D. Gemperle, *Clin. Chim. Acta* **19**, 477 (1968).

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Factors Influencing the Uptake of Vitamin B_{12} by Normoblastic and Vitamin B_{12}-Deficient Bone Marrow Cells

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Abstract. The uptake of serum-bound ^{57}Co-B_{12} by freshly aspirated human bone marrow cells is an active, calcium-dependent process requiring the presence of cellular respiration, oxidative phosphorylation and free sulphydryl groups. In contrast, serum-mediated uptake of ^{57}Co-B_{12} by reticulocytes and erythrocytes has been reported to be an energy-independent, surface-adsorption phenomenon. Bone marrow cells also take up ^{57}Co-B_{12} in the absence of serum. Such uptake is dependent on cellular respiration but not on calcium ions or free sulphydryl groups. The average value for the uptake of serum-bound ^{57}Co-B_{12} by vitamin B_{12}-deficient bone marrow cells was 2.7 times greater than that for normoblastic marrow cells.

Key Words
Bone marrow cells
Metabolic inhibitors
Vitamin B_{12} deficiency
Vitamin B_{12} uptake

Several studies have shown that a protein present in normal serum promotes the uptake of vitamin B_{12} by mammalian cells [1, 2, 4, 5, 6]. In the majority of these investigations, tumour cells or non-dividing cells such as human reticulocytes have been used. The characteristics of vitamin B_{12} uptake by rapidly dividing, non-malignant cells such as haemopoietic cells have not yet been adequately studied. In this paper we present data on the factors influencing vitamin B_{12} uptake by cells from normoblastic and vitamin B_{12}-deficient megaloblastic marrow aspirates. In addition, the recent observation that cell suspensions prepared from vitamin B_{12}-deficient human bone marrow take up more radioactive vitamin B_{12} than cells from normoblastic marrow [8] has been investigated in a larger number of patients.

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Materials and Methods

Freshly aspirated bone marrow was mixed with 5 ml Hanks' solution containing preservative-free heparin. The marrow aspirate was forced through a 21-gauge needle once and a 23-gauge needle twice, and the resulting cell suspension centrifuged at 1,100 *g* for 5 min. The buffy coat was separated, washed 3 times in Hanks' solution and finally resuspended in 4–8 ml of Hanks' solution. The nucleated cell count and red cell count were determined using a Coulter counter (Model S).

^{57}Co-labelled cyanocobalamin (^{57}Co-B_{12}; 15–60 μCi/μg) was obtained from the Radiochemical Centre, Amersham. Uptake of ^{57}Co-B_{12} was determined using a modification of the method of RETTEY *et al.* [6]. To 1 ml of the marrow cell suspension was added 1 ml Hanks' solution, 2 ml of 0.9% NaCl containing 10 mM $CaCl_2$ and 1 ml of autologous serum pre-incubated with 1 ng ^{57}Co-B_{12}. The use of Hanks' solution instead of NaCl-$CaCl_2$ was later shown not to affect uptake. 1 ng of ^{57}Co-B_{12} per ml serum has been shown to be a suitable subsaturating dose in previous studies [6, 8]. The mixture was incubated at 37 °C in a shaking water bath. After the appropriate time interval, the cells were washed 3 times in ice-cold 0.9% saline and lysed in 2 ml of distilled water. The uptake of ^{57}Co-B_{12} was measured by counting the lysate in a Packard scintillation counter. In the majority of the experiments, each culture bottle contained 0.8–2.0 $\times$ 10^7 nucleated cells and less than 0.5 $\times$ 10^9 erythrocytes. Duplicate cultures were frequently set up using 1 ml Hanks' solution or 1 ml 0.9% NaCl instead of autologous serum. Uptake of ^{57}Co-B_{12} under these conditions is described as saline-mediated uptake in this paper.

As marrow suspensions contain many more mature erythrocytes than nucleated marrow cells, the contribution of erythrocyte uptake to the total uptake in marrow cultures had to be estimated. For this purpose, 4–6 different dilutions of washed red cells were prepared from each of 6 healthy individuals and the serum-mediated uptake by 1 ml of each red cell suspension determined. The effect of 5 mM 2-iodoacetamide on red cell uptake was also studied.

The effects of temperature and a number of chemicals on the uptake of ^{57}Co-B_{12} by bone marrow cells were also investigated. To study the influence of various metabolic poisons, sufficient Hanks' solution was added to 1 ml of the marrow suspension to make a total volume of 4 ml after the addition of the chemical under study. The pH of this mixture was adjusted to 7.4 when necessary. Marrow cells were pre-incubated with the metabolic inhibitor for 15–30 min at 37 °C before the addition of 1 ml serum containing 1 ng ^{57}Co-B_{12} and further incubation at 37 °C for 60–90 min.

Results

Figure 1 shows the results of experiments designed to study serum-mediated vitamin B_{12} uptake by varying numbers of mature erythrocytes. The buffy coat-poor normal erythrocyte suspensions were pre-

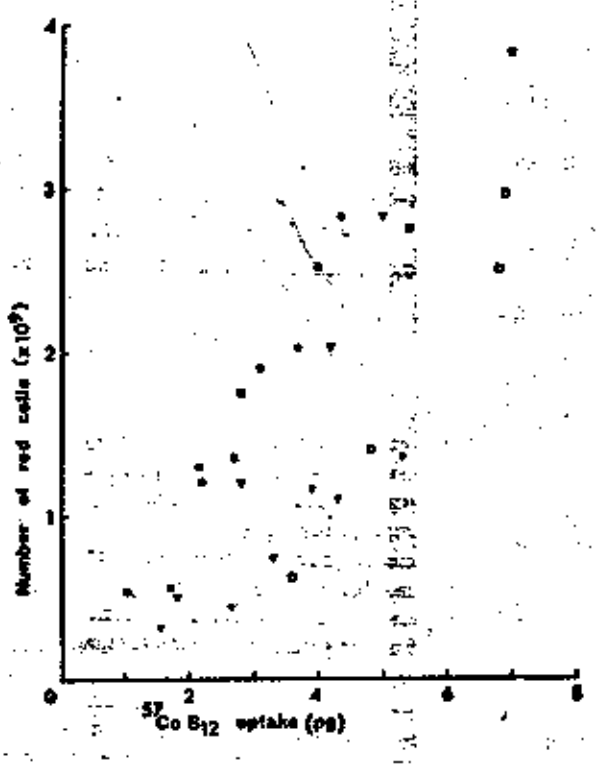

Fig. 1. Serum-mediated uptake of ^{57}Co-B_{12} by varying numbers of normal erythrocytes.

pared from venous blood and contained less than 1.5% of reticulocytes. It is evident that there is a linear relationship between ^{57}Co-B_{12} uptake and erythrocyte numbers, and that 10^9 red cells may take up to 4.2 pg (range 1.7–4.2 pg) of vitamin B_{12}. The average uptake by 0.5×10^9 red cells calculated from these data was 1.7 pg. The transfer of serum-bound ^{57}Co-B_{12} to erythrocytes was not inhibited by 5 mM iodoacetamide.

Figure 2 shows the uptake of ^{57}Co-B_{12} by cell suspensions from 15 normoblastic and 9 vitamin B_{12}-deficient marrows, after 90 min incubation at 37 °C. Cell suspensions prepared from normoblastic marrows contained an average of 0.36×10^9 erythrocytes per 10^7 nucleated cells and those prepared from B_{12}-deficient marrows contained 0.21×10^9 erythrocytes per 10^7 nucleated cells. The average value for serum-mediated uptake by cells from normoblastic marrows was 3.1 pg per 10^7 nucleated cells and the corresponding value for B_{12}-deficient marrows, 8.4 pg per 10^7 nucleated cells. There is some overlap between the two

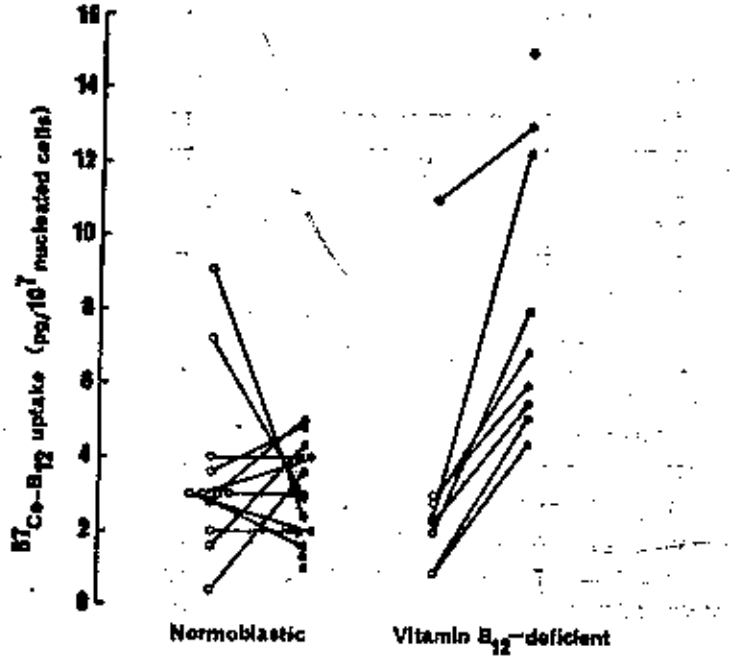

Fig. 2. Uptake of ^{57}Co-B_{12} by marrow suspensions. ○ = Saline-mediated uptake; ● = serum-mediated uptake. Lines connect results for the same marrow.

groups, caused by the inclusion of two mildly megaloblastic marrow aspirates in the study.

Saline-mediated uptake by cells from normoblastic marrows varied widely, being higher than, equal to, or less than serum-mediated uptake. Saline-mediated uptake was always less than serum-mediated uptake in the cultures from vitamin B_{12}-deficient marrow aspirates. The average value for saline-mediated uptake was 3.5 pg per 10^7 nucleated cells in the normoblastic group and 3.2 pg in the vitamin B_{12}-deficient group. The ratio between serum-mediated and saline-mediated uptake was 0.9 in the normoblastic group and 2.6 in the megaloblastic group.

Figure 3 shows the rate of uptake of ^{57}Co-B_{12} by marrow cells over a 45–90 min incubation period. Transfer of serum-bound ^{57}Co-B_{12} to marrow cells was initially very rapid and gradually diminished thereafter. Serum-mediated uptake at 90 min was 1.8–2.4 times the value at 5 min in normoblastic cultures and 1.5–2.7 times that at 5 min in vitamin B_{12}-deficient cultures.

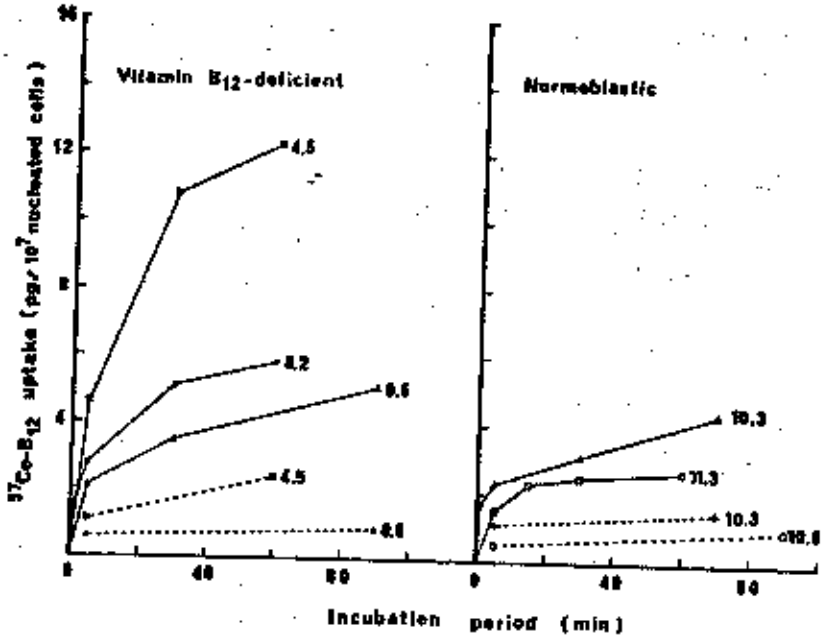

Fig. 3. Rate of uptake of ^{57}Co-B_{12} by vitamin B_{12}-deficient and normoblastic marrow suspensions. ——— = Serum-mediated uptake; - - - - = saline-mediated uptake. The numbers opposite each curve are haemoglobin values in g/100 ml.

The effect of temperature on serum-mediated uptake was similar for normoblastic and vitamin B_{12}-deficient marrows. Uptake of ^{57}Co-B_{12} at 4 °C was 30% lower than that at 37 °C, after a 20 min incubation period.

Table I shows the effects of various metabolic inhibitors on the serum-mediated transfer of ^{57}Co-B_{12} to marrow cells. The results were similar in cultures from normoblastic and vitamin B_{12}-deficient marrows and are, therefore, not given separately. The inhibition of uptake seen with disodium ethylenediamine tetra-acetate (Na_2EDTA), sodium cyanide, 2-iodoacetamide and 2,4-dinitrophenol does not result from cell death caused by these metabolic inhibitors as dead bone marrow cells take up more vitamin B_{12} than living cells. Thus, marrow cells killed by fixation in absolute methanol for 10 min took up approximately 4–5 times more serum-bound ^{57}Co-B_{12} than living cells. Serum-mediated uptake of ^{57}Co-B_{12} was increased to a similar extent in the presence of 10% formalin.

Table I. Effect of metabolic inhibitors on serum-bound ^{57}Co-B_{12} uptake by bone marrow cells (normoblastic and vitamin B_{12}-deficient)

Concentration M	Inhibitor	Inhibition[1] %
5×10^{-3}	NaCN	39.2
5×10^{-4}	2,4-dinitrophenol	16.1
5×10^{-3}	2-iodoacetamide	37.9
10^{-6}	Na_2EDTA	78.4
10^{-3}	Na_2EDTA + $CaCl_2$	0.0
1.5×10^{-6}		
10^{-3}	NaF	1.0
10^{-3}	$NaHAsO_4$	0.0

[1] Each value represents the mean of 6–10 experiments.

Saline-mediated uptake of ^{57}Co-B_{12} by bone marrow cells was unaffected by Na_2EDTA, 2-iodoacetamide and 2,4-dinitrophenol but was depressed by 44.9% in the presence of 50 mM sodium cyanide.

Discussion

The average value for serum-mediated uptake by normoblastic marrow cultures incubated for 90 min at 37 °C was 3.1 pg ^{57}Co-B_{12} per 10^7 nucleated marrow cells. The data on normal erythrocytes indicate an average uptake of 1.7 pg per 0.5×10^9 red cells. As an average of 0.36×10^9 erythrocytes were present per 10^7 nucleated marrow cells, not more than 50% of the 3.1-pg uptake could be attributed to uptake by red cells. That the larger number of erythrocytes does not mask uptake by 10^7 nucleated marrow cells is supported by the finding of progressively increasing uptake in normoblastic marrow cultures, over a 90-min incubation period. Whereas most of the uptake by erythrocytes occurs within the first 5 min of incubation [6], uptake by normoblastic marrows was nearly twice as much at 90 min as at 5 min.

Preliminary studies indicate that the serum-mediated uptake of ^{57}Co-B_{12} by macrocytes is similar to that by normal erythrocytes [unpublished observations]. Thus, reticulocyte-poor red cell suspensions from 5 vitamin B_{12}-deficient patients showed an uptake of 2.1–3.5 pg/10^9

erythrocytes. The contribution of erythrocyte uptake to the total uptake in marrow cultures would, therefore, be similar for both normoblastic and vitamin B_{12}-deficient marrow cultures.

The earlier finding of higher serum-mediated uptake by vitamin B_{12}-deficient marrow cultures, compared to normoblastic cultures [8], has been confirmed in a larger number of patients. However, patients with mildly megaloblastic marrows and haemoglobin levels within or near the normal range, either showed only slightly increased serum-mediated vitamin B_{12} uptake, or uptake within the normoblastic range. The average value for saline-mediated uptake was similar in the normoblastic and vitamin B_{12}-deficient groups.

Serum-mediated transfer of ^{57}Co-B_{12} to cells from normoblastic and vitamin B_{12}-deficient marrows showed several similarities. Uptake was progressive over a 90-min period, sensitive to changes in temperature, and was markedly inhibited by 10^{-2} M Na_2EDTA. Inhibition by Na_2 EDTAwas corrected by the addition of 1.5×10^{-2} M calcium chloride, indicating that uptake was dependent on the presence of calcium ions. The transfer of ^{57}Co-B_{12} to marrow cells was appreciably depressed in the presence of 50 mM sodium cyanide, indicating that uptake was dependent upon cellular respiration. Iodoacetamide (5 mM) also caused significant inhibition, presumably due to combination with sulphydryl groups [5] of a hitherto unidentified enzyme or intracellular transport protein which is necessary for normal uptake. It is unlikely that the latter effect is due to inhibition of glycolysis, as two other inhibitors of glycolysis, sodium fluoride and sodium arsenate, were without effect.

Paranchych and Cooper [5] found that uptake by Ehrlich ascites cells was only partly inhibited by 0.1 mM 2,4-dinitrophenol (an inhibitor of oxidative phosphorylation) and concluded that ATP was not directly involved in vitamin B_{12} uptake. In the present study, the uptake of serum-bound ^{57}Co-B_{12} by bone marrow cells was significantly, although only slightly, affected in the presence of 5 mM dinitrophenol. In contrast, the uptake of serum-bound ^{57}Co-B_{12} by HeLa cells was strongly inhibited by 5 mM dinitrophenol [3]. These differences probably reflect variations in metabolic activity in different cell types. For example, ATP requirements and intracellular ATP stores may vary considerably in these three cell types leading to different rates of depletion of ATP when oxidative phosphorylation is uncoupled.

The transfer of serum-bound vitamin B_{12} to bone marrow cells (normoblastic or vitamin B_{12}-deficient) is, therefore, an active, energy-de-

pendent process, differing from vitamin B_{12} uptake by human reticulocytes (and erythrocytes) which appears to be largely an energy-independent surface adsorption phenomenon [6]. As vitamin B_{12} is required for DNA synthesis during cell proliferation [7], anucleate reticulocytes would be expected to have little requirement for it. No significant differences between marrow cells and tumor cells were detected [3, 5].

Two phases have been distinguished in the uptake of ^{57}Co-B_{12} by Ehrlich ascites cells: an energy-independent primary uptake phase which is complete in less than 1 min, followed by an energy-dependent secondary uptake phase [5]. The primary uptake phase was considered to represent a physico-chemical adsorption of serum-bound cyanocobalamin to receptors at the cell surface. We made no attempt to distinguish between primary and secondary uptake in marrow cultures, as primary uptake by the small number of nucleated cells would have been obscured by that due to the larger number of erythrocytes. Marrow suspensions virtually free of mature erythrocytes will be required for such studies. Nevertheless, the observation that iodoacetamide does not inhibit serum-mediated uptake by erythrocytes whereas it does depress uptake by bone marrow cells suggests that primary uptake is not dependent on the presence of free sulphydryl groups.

Saline-mediated uptake of ^{57}Co-B_{12} by bone marrow cells was partially inhibited by 50 mM sodium cyanide, indicating a dependence on cellular respiration. However, unlike serum-mediated uptake, saline-mediated uptake was not dependent on calcium ions or free sulphydryl groups. Evidently, two different mechanisms are involved in the uptake of serum-bound and free ^{57}Co-B_{12}. As vitamin B_{12} is normally bound to specific transport proteins in the serum, it is likely that only serum-mediated uptake is of physiological importance. Saline-mediated uptake is primarily a laboratory artefact but may be operative *in vivo* when there is an excess of unbound vitamin B_{12}.

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The absorption of [^{58}Co]cyanocobalamin by unweaned rats

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1. [^{58}Co]cyanocobalamin was given by stomach tube to Wistar albino rats aged between 6 h and 24 days. The fraction of the dose absorbed was assessed by measuring the amount retained by the animals after 7 days, by total body counting.

2. Rats up to 4 days old absorbed about 90% of the test dose, compared with a mean of 83% at the age of 8 days, 66% at 16 days and 47% at 24 days.

3. The proportion of the test dose absorbed did not appear to be affected by starvation for up to 24 h, by increasing the dose from 0·05 ng to 0·25 ng/g body-weight, or by giving the [^{58}Co]cyanocobalamin mixed with rat's milk.

4. The results support earlier suggestions that newborn rats absorb vitamin B_{12} by a different mechanism from that in adults.

During attempts in this Department to produce deficiency of vitamin B_{12} in rats by dietary depletion, it became necessary to breed from rats already receiving a deficient diet. It therefore seemed to be of interest to attempt to determine what proportion of the available vitamin B_{12} was absorbed by young rats. Our results show that rats up to 4 days old absorb about 90% of a test dose of [^{58}Co]cyanocobalamin, but the absorption decreases to adult levels by 24 days. Doass & Wilson (1963) had previously shown that intestinal segments from foetal and newborn rats took up much more [^{57}Co]vitamin B_{12} than similar segments from adult rats, but the uptake declined towards adult levels by about 14 days. The stomach wall of foetal and newborn rats contained much less intrinsic factor than that of adult rats, though the levels increased rapidly after the 1st week of life.

EXPERIMENTAL

Animals. Young rats were bred in the Department from albino parents of the Wistar strain, which were fed *ad lib.* on a diet of standard rat cake (modified diet 41B; Herbert C. Styles (Bewdley) Ltd).

Administration of [^{58}Co]cyanocobalamin and management of animals. The young rats were left with their mothers until just before dosing, when they were marked and weighed. An aqueous solution of [^{58}Co]cyanocobalamin (Radiochemical Centre, Amersham, Bucks; specific activity 40 $\mu c/\mu g$) was given through a fine polythene stomach tube, the quantity being adjusted so that each rat received 0·05 ng cyanocobalamin/g body-weight. This dose gave satisfactory counts of radioactivity even in very small rats. It was chosen because Taylor, Mallett, Witts & Taylor (1958) showed that in adult rats weighing 200–250 g there was a linear relationship between the amount of cyanocobalamin absorbed and the dose, in the range 1–10 ng. In a few experiments (Table 4) five times as much cyanocobalamin was given. In experiments to study the effect of milk on absorption (Table 5), [^{58}Co]cyanocobalamin of specific

activity 150 $\mu c/\mu g$ was mixed with rat's milk, the vitamin B_{12} content of which had been measured by microbiological assay with *Lactobacillus leichmannii*. The total vitamin B_{12} content of the mixture was calculated from the assay results and the amount of [^{58}Co]cyanocobalamin used, and the rats received 0·05 ng total vitamin B_{12} activity/g body-weight.

After dosing and counting, the young rats were returned to their mothers and weighed daily. It was found that animals in which the oesophagus was damaged during dosing did not grow satisfactorily. Any which failed to gain weight daily were therefore discarded. Whenever possible, different members of each litter were dosed at different ages to allow for possible variation between litters in ability to absorb vitamin B_{12}. There was no evidence of transfer of ^{58}Co from treated to untreated litter-mates.

Measurement of radioactivity. Radioactivity in the rats was counted by placing each animal in a small cardboard box resting on a polythene block in the plastic phosphor well scintillation counter described by Warner & Oliver (1962). A radioactive standard containing a known amount of the dosing solution, made up to 4 ml with water in a plastic tube, was also counted. The radioactivity was counted immediately after the rats had been dosed and 7 days later. The fraction of the dose retained after 7 days was taken as the amount absorbed. In some experiments the radioactivity was also counted at intervals before the 7th day.

RESULTS

Retention of isotope after oral doses of [^{58}Co]cyanocobalamin. Preliminary experiments (Table 1) showed that in rats dosed at or before 8 days of age there was an initial loss of isotope before the 5th day, with little or no further loss between the 5th and

Table 1. *Percentage of test doses of [^{58}Co]cyanocobalamin retained by rats at different times after dosing*

Age when dosed (days)	No. of observations	% of test dose retained on: 5th day Mean	5th day Standard deviation	7th day Mean	7th day Standard deviation
0·25	20	90·7	4·3	90·8	4·3
1	3	94·0	5·2	87·0	3·0
8	15	88·7	5·4	86·0	4·8
24	9	63·1	8·8	56·0	3·8

7th days. In 24-day-old rats the early loss was larger, with some excretion continuing between the 5th and 7th days, although this was much smaller than before the 5th day and was not significant on the basis of these nine observations ($t = 2·02$, $0·1 > P > 0·05$). These results suggested that by using the fraction of the dose retained after 7 days as a measure of the amount absorbed, we obtained an accurate assessment of absorption in rats up to 8 days old, but possibly a slight over-estimate

in older animals. This error, however, would decrease rather than magnify the differences shown by our results.

Absorption of [^{58}Co]cyanocobalamin at different ages. The 323 observations on rats aged from 6 h to 24 days show that during the first 4 days of life the animals absorbed on average about 90 % of a test dose of [^{58}Co]cyanocobalamin (Table 2). The mean absorption fell to 83 % in animals aged 8 days, to 66 % in those aged 16 days and to only 47 % in those aged 24 days. The results for the older animals showed a wider scatter than those for the younger ones.

Table 2. *Percentage of test doses of [^{58}Co]cyanocobalamin (0·05 ng/g body-weight) absorbed by rats of various ages*

Age when dosed (days)	No of observations	% of test dose absorbed		
		Mean	Standard deviation	Range
0·25	42	89·9	6·0	78–100
0·5	17	89·5	7·6	75–100
1	61	92·1	6·4	75–100
2	41	86·8	5·5	67–100
4	45	88·2	7·4	61–100
8	40	82·7	8·5	56–98
16	32	66·0	15·4	29–86
24	45	46·8	17·8	11–96

Table 3. *Percentage absorption of test doses of [^{58}Co]cyanocobalamin by rats of different ages, after starvation, compared with that by their unstarved litter-mates*

Age when dosed (days)	Period of starvation (h)	No of litters studied	% of test dose absorbed by					
			Starved rats			Unstarved rats		
			No. of animals	Mean	Standard deviation	No. of animals	Mean	Standard deviation
1	6	4	16	90·2	8·6	14	93·0	4·3
	12	3	12	90·5	6·6	10	89·8	7·0
4	6	1	3	92·0	3·5	3	92·3	3·2
	12	3	12	90·0	2·9	11	86·6	5·7
8	12	2	8	88·1	2·9	8	84·2	5·6
	24	1	3	81·0	2·6	3	79·3	1·2
16	18	2	7	66·4	14·9	5	49·2	11·3
	24	1	2	72·0	—	2	56·0	—

Effect of starvation. All the results summarized in Table 2 were obtained with animals that had been with their mothers until just before the [^{58}Co]cyanocobalamin was given. It was thought that to starve such young animals before dosing would be difficult and might be fatal. To see whether the presence of food in the stomach affected absorption, some rats were removed from their mothers for periods up to 24 h before the cyanocobalamin was given. Their litter-mates were left with the mothers and were dosed at the same time. The results (Table 3) show that fasting had no apparent effect on absorption in rats dosed when less than 8 days old. In 16-day-old rats the results were highly variable, as was found in the main study with

rats of this age. However, there was no significant difference between the results for 16-day-old rats starved for 18 h and those for the corresponding unstarved animals ($t = 2{\cdot}17$, $0{\cdot}1 > P > 0{\cdot}05$).

Absorption of [^{58}Co]cyanocobalamin from doses of 0·25 ng/g body-weight. The effect of giving five times more [^{58}Co]cyanocobalamin than was used in the main study was investigated in a few animals aged between 6 h and 16 days. The results (Table 4) were all similar to those found in animals of corresponding ages receiving 0·05 ng cyanocobalamin/g body-weight (Table 2).

Effect on absorption of giving [^{58}Co]cyanocobalamin mixed with rat's milk. The results (Table 5) show that the proportions of the test dose absorbed when the vitamin was mixed with rat's milk were similar to those absorbed by animals of similar ages when the cyanocobalamin was in aqueous solution.

Table 4. *Percentage of test doses of [^{58}Co]cyanocobalamin (0·25 ng/g body-weight) absorbed by rats of various ages*

Age when dosed (days)	No. of observations	% of test dose absorbed	
		Mean	Range
0·25	6	92·2	84–97
0·5	3	93·3	93–94
1	8	91·0	89–97
8	3	79·3	78–80
16	2	56·0	—

Table 5. *Percentage of test doses of [^{58}Co]cyanocobalamin, mixed with rat's milk, absorbed by rats of different ages (each animal received 0·05 ng total vitamin B_{12} activity/g body-weight)*

Age when dosed (days)	No. of observations	% of test dose absorbed	
		Mean	Range
4	5	93·5	91–96
8	6	87·9	83–93
16	6	72·4	66–80
24	6	55·0	48–60

DISCUSSION

In order to interpret our results we have made the usual assumption that the distribution of ^{58}Co represents the distribution of [^{58}Co]cyanocobalamin. According to Boass & Wilson (1963), little or no intrinsic factor is produced by the rat stomach during the 1st week of life. Our results show that during this period rats absorb about 90% of a test dose of [^{58}Co]cyanocobalamin, presumably by a mechanism independent of intrinsic factor. Although it is difficult to compare results for isolated segments of intestine with those for intact animals, this high absorption is in agreement with the high uptake by intestinal segments of newborn rats (Boass & Wilson, 1963). After the 1st week the absorption of cyanocobalamin decreases and probably becomes increasingly dependent on intrinsic factor. By the 24th day the mean absorp-

tion had fallen to levels found in adult rats by other authors, e.g. 33·5% (Watson & Florey, 1955), 42·9% (Clayton, Latner & Schofield, 1957), 52·3% (Taylor *et al.* 1958 and 57% (Smith & Ellis, 1965).

A possible criticism of our results with very young rats might be that owing to the technical difficulty of intubating the stomach of such small animals, the test dose might be introduced into the tissues rather than into the stomach. We feel that this is unlikely, because results were only included from those rats which grew normally after dosing. Also, when the wall of the oesophagus was accidentally pierced, the catheter could be seen under the skin and was usually stained with blood when removed. If the dose entered the lungs it was expectorated by the rat. Animals in which such abnormalities were noted were discarded.

The reason for the greater variation of the results in older rats as compared with those in younger animals is not clear. The results of Boass & Wilson (1963) suggest that considerable variations occur in the amount of intrinsic factor secreted by rats aged between 7 and 17 days. Thus the change in the mechanism of absorption of cyanocobalamin from being independent of intrinsic factor to dependence on intrinsic factor may occur at different ages in different individuals. This could account for the variability. Likewise, we can only offer speculative explanations for the absorption of less than 20% of the test dose by some 24-day-old rats.

Absorption does not appear to be affected by starvation (Table 3) or by giving the cyanocobalamin with rat's milk rather than in aqueous solution (Table 5). Rats up to 16 days old appear to absorb 0·25 ng cyanocobalamin/g body-weight as efficiently as they absorb 0·05 ng/g (Table 4). This is in agreement with the results of Smith & Ellis (1965), who found that adult rats weighing 250–300 g absorbed roughly the same proportion of doses of either 10 or 90 ng cyanocobalamin.

The milk of rats on stock diets contains up to 100 ng/ml of vitamin B_{12} activity for *Lactobacillus leichmannii* (D. L. Williams & G.H. Spray, unpublished observations). The present results suggest that most of this vitamin B_{12} is absorbed by baby rats until they are at least a week old, assuming that the absorption of [^{58}Co]cyanocobalamin reflects that of vitamin B_{12} from milk. The normal absorption of cyanocobalamin when given with rat's milk, supports this assumption. To try to obtain further information, nursing mothers were injected with [^{58}Co]cyanocobalamin and the radioactivity in their litters was counted afterwards. The young rats did not take up sufficient isotope for accurate counting until 3 days after the mothers were injected, so that the results could not be compared with those already described.

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EFFECT OF INSULIN ON THE TRANSPORT OF VITAMIN B_{12} INTO THE PERFUSED RAT LIVER **

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Head: Prof. G. B. J. Glass M. D.

The effect of insulin on the Vit. B_{12} transport into the perfused liver of rats was studied. $^{57}CoB_{12}$ accumulates in the liver tissues against gradient concentration. Insulin added to perfusate increased significantly the rate of uptake of the $^{57}CoB_{12}$ into the liver tissue, the differences being significant at 60 minutes, and gradually disappearing at the end of 3 hours.

In previous investigations, one of us (K.W.) found that administration of insulin with certain drugs increased the tissue content and pharmacological effects of these drugs [1, 8—10]. This was also seen when insulin was given together with glucose which prevented post-insulin hypoglycemia. Opposite effects were observed in alloxan diabetes [11, 12].

It was also found [13] in studies on perfused rat liver that insulin increased the transport of isonicotinic acid hydrazide from perfusate into the liver tissue ***. Since the transport of this foreign to the liver substance into the liver tissue is limited, only small amounts of this drug were found in the liver. The augmentation of the transport of the isonicotinic acid hydrazide into the liver by insulin could be observed only when the concentration of the drug in the perfusate was sufficiently high.

Because vitamin B_{12} is a normal component of the liver and is normally stored there at a concentration of about 1 ng/gm tissue [2] in this study we examined the effect of insulin on the transport of ^{57}Co-labelled vitamin B_{12} into the perfused rat liver.

MATERIALS AND METHODS

The experiments were carried out on 6 Wistarstrain male rats, weighing 250—320 g, and fasting for 18 hours, who acted as liver donors.

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The Haft [3] modification of Miller [5] liver perfusion technique was used. The livers were perfused with a mixture of 80—100 ml of blood freshly drawn from Sprague Dawley retired breeder rats, and one-third its volume of Ringer's solution containing 45 mg heparin. The recirculating perfusate was continuously oxygenated with 95% O_2+5% CO_2.

$^{57}CoB_{12}$ ** at the dose of 0·005 μg (0·033 μc/ml) with or without insulin (Lilly) at the dose of 0·01 U/ml was added to the perfusate. The concentration of radio-B_{12} in the liver, bile and perfusate was determined by radioactivity counting using a γ spectrometer with a NaI-Thalium crystal at 1, 10, 30, 60, 120 and 180 minutes after the perfusion was started. The statistical evaluation was done by Student's „t" test.

RESULTS

$^{57}CoB_{12}$ accumulated in the liver against a concentration gradient. After 120 and 180 minutes of perfusion, the concentration of $^{57}CoB_{12}$ in the liver tissue was higher than in the perfusate.

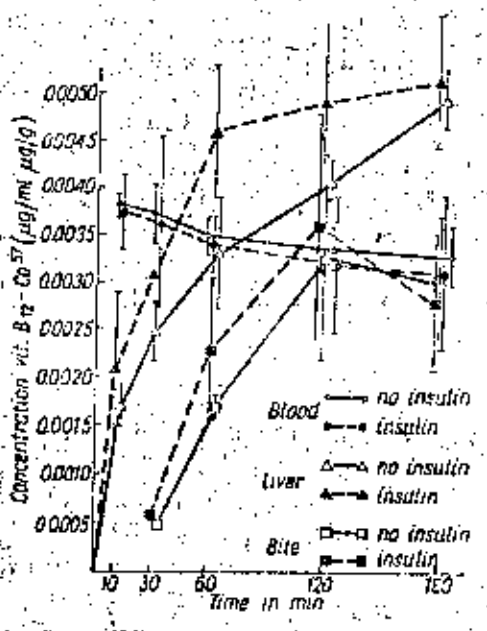

Fig. 1. The influence of insulin on $^{57}CoB_{12}$ transport into the perfused liver. Each point is the mean value of 6 experiments. The vertical lines indicate the range of values recorded in all experiments.

When insulin was added to the perfusate, the mean concentration of the $^{57}CoB_{12}$ in the liver increased. While in control experiments, after 60 minutes of perfusion, the concentration of $^{57}CoB_{12}$ was 3·34±0·58 ng/g, after 60 minutes of perfusion with insulin it was 4·54±0·7 ng/g. (Fig. 1). This difference was

** Generously supplied by Dr. Elmer Albert, Medical Director, Merck Sharpe & Dohme Co., Westpoint, Pa.

statistically significant with the p value less than 0·02. The concentration of $^{57}CoB_{12}$ in the liver at 60 minutes of perfusion in experiments with insulin was as high as that 180 minutes after perfusion with $^{57}CoB_{12}$ without insulin. At 120 minutes of perfusion, the mean vitamin B_{12} concentration in the liver with insulin was higher than in controls without insulin; because of large standard deviation, this difference was not statistically significant, however. The difference disappeared in three hours.

The excretion of $^{57}CoB_{12}$ in the bile was also higher in perfusion experiments with insulin than without it. However, the differences in the concentration of $^{57}CoB_{12}$ in bile with and without insulin were statistically not significant. This was possibly due to low concentration of $^{57}CoB_{12}$ in the bile which was in the range of 0·2—0·4 per cent of administered dose.

COMMENT

It appears from these investigations that insulin increases, at one hour, the uptake of $^{57}CoB_{12}$ from the perfusate into the rat liver. Other authors [4] using different methods also found that insulin increased accumulation of vitamin B_{12} in the liver tissue.

In our as yet unpublished work we have found that the uptake of $^{57}CoB_{12}$ by liver slices incubated with radio-B_{12} in bicarbonate buffer for 90 minutes also was higher in the presence of insulin than in the controls. This effect was seen in all seven experiments and was statistically significant.

In this context it should be mentioned that insulin has an in vivo effect also on the circulating endogenous vitamin B_{12}. In the work of Rosenthal, et al. [6] from our laboratory, hypoglycemic doses of insulin induced increased serum Vit. B_{12} levels in 5 of 6 subjects tested. This was prevented by oral glucose loading. There is some evidence that this effect is mediated by the secretion of growth hormone resulting from insulin hypoglycemia [6, 7].

Thus, insulin has a complex „transport effect" upon the shifts of B_{12}. It has a certain though limited stimulating effect upon the membrane transport of the exogenous vitamin B_{12} into the perfused liver in vitro which results in the enhancement of the hepatic uptake of the vitamin. On the other hand, in vivo through hypoglycemia and most probably growth hormone discharge, it also causes a increase of the endogenous vitamin B_{12} circulating in the plasma. These two insulin effects may play a role in the shifts of vitamin B_{12} between the blood and liver under physiological conditions.

K. Wiśniewski, G. B. J. Glass

WPŁYW INSULINY NA TRANSPORT WITAMINY B_{12} DO PERFUNDOWANEJ WĄTROBY SZCZURA

Streszczenie

Przeprowadzono badania nad wpływem insuliny na transport witaminy B_{12} do perfundowanej wątroby szczura.

$^{57}CoB_{12}$ nagromadzał się w tkance wątrobowej przeciw gradientowi stężeń. Insulina podana do perfuzatu zwiększała szybkość nagromadzania witaminy $^{57}CoB_{12}$ w tkance wątrobowej. Największe różnice w zawartości $^{57}CoB_{12}$ w wątrobie perfundowanej z insuliną i bez insuliny stwierdzano w 60 minucie doświadczenia.

K. Wiśniewski, G. B. J. Glass

ВЛИЯНИЕ ИНСУЛИНА НА ТРАНСПОРТ ВИТАМИНА B_{12} В ПЕРФУНДИРОВАННУЮ ПЕЧЕНЬ КРЫСЫ

Содержание

Проведены исследования над влиянием инсулина на транспорт витамина B_{12} в перфундированную печень крысы.

$^{57}CoB_{12}$ сконцентрировался в печеночной ткани против градиенту концентраций. Инсулин, введенный в перфузат увеличивал быстроту концентраций. Инсулин, введенный в перфузат увеличивал быстроту магазинирования витамина $^{57}CoB_{12}$ в печеночной ткани. Самые большие различия в содержании $^{57}CoB_{12}$ в перфундированной печени с инсулином и без него обнаружили на 60 минуте. эксперимента.

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Study of One-carbon Metabolism in Neonatal Vitamin B_{12}-deficient Rats [1,2]

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ABSTRACT By feeding diets deficient in vitamin B_{12} and other lipotropic substances, anomalies such as hydrocephalus, umbilical hernia and spina bifida were produced in neonatal rats. The incidence of congenital hydrocephalus was increased when 2.1 or 4.2 mmoles/100 g diet of DL-methionine was added to diets deficient in choline and vitamin B_{12}. Measurements of DNA, RNA, protein, proteolipid, and total lipid showed no quantitative differences between embryos born to dams maintained on deficient or control rations. Likewise, there were no differences found in brain phospholipids or in the incorporation of L-methionine-methyl-^{14}C into various chemical constituents. It was concluded that alterations in brain phospholipids did not play a significant role in the pathogenesis of the nutritionally induced neural developmental anomalies, and it is suggested that dams furnish one-carbon fragments to the developing embryos at the expense of their own tissue requirements.

Deficiencies of vitamin B_{12} or folic acid during gestation result in congenital hydrocephalus in neonatal rats (1, 2). Likewise, rats born to dams fed a ration low in folic acid produce offspring which, if fed this same ration until maturity, show inferior maze-learning abilities. It has been suggested that brain alterations occur before birth and subsequent nutritional deficiency has little if any effect on maze learning (3). The nutritional quality of the mother's diet may have a direct effect upon the biochemical content of the fetus. For example, female rats fed an 8% protein diet for 1 month before mating and throughout gestation gave birth to young whose brains contained significantly less deoxyribonucleic acid (DNA) and protein than did progeny from dams fed a 27% protein diet (4). It has been suggested that the qualitative biochemical changes may constitute the basis for the frequently reported impaired behavior of the offspring from protein-deprived mothers. Bruemmer et al. (5) reported that 1-day-old offspring from vitamin B_{12}-deficient rats had higher DNA concentrations per gram of brain tissue, and ribonucleic acid (RNA) concentration per cell was lower in B_{12}-deficient offspring (5).

Morphologic alterations in neonatal animals indicate that hydrocephalus induced by vitamin B_{12} and that induced by folic acid deficiency are similar (6, 7). Hydrocephalus is caused by stenosis of the cerebral aqueduct and is associated with aplasia of the subcommissural organ, pineal gland and other neural structures. The basic defect is thought to be concerned with the multiplication, migration or maturation of primitive neural elements. A number of dietary factors other than vitamin B_{12} have been shown to influence the incidence or severity of lesions. The deletion of choline from a vitamin B_{12}-deficient diet increased the incidence of abnormalities in neonatal rats, and methionine in amounts equivalent to choline in methyl groups did not offer the same protective effects (8). A synergistic action was also found when X-methyl folic acid was added to a vitamin B_{12}-deficient diet (8). This interrelation between developmental neural abnormalities and the role of vitamin B_{12} in one-carbon metabolism has been confirmed by O'Dell (9).

Because it has been shown that depleting the dam of lipotropic agents increases the incidence of developmental anomalies (8), and because liver and plasma phospholipids are known to be reduced by a deficiency of choline (10), this experiment was designed to investigate the role

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[2] This material was presented, in part, at the 52nd Annual Meeting of the Federation of American Societies for Experimental Biology, April, 1968.

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of one-carbon metabolism in the pathogenesis of nutritionally induced hydrocephalus. The purposes of this experiment were: 1) to determine whether any biochemical alterations could be detected in brains or livers from neonatal rats born to dams maintained on diets deficient in vitamin B_{12} or other lipotropic agents, and 2) to determine if methionine can prevent the enhancing effects choline deficiency has on the induction of hydrocephalus.

METHODS

Female weanling albino rats of the Caesarean Derived (CD), Sprague-Dawley strain were obtained commercially.[3] The animals were housed in air-conditioned animal quarters; food and water were supplied ad libitum. Three female animals were housed together in large, raised, screen-wire cages and fed either a vitamin B_{12}-deficient diet or a vitamin B_{12}-supplemented diet during the growth period. The 20% protein diet shown in table 1 was supplemented with 0.18% choline bitartrate and 0.32% DL-methionine during the growth period; vitamin B_{12} was added to the control diet at the rate of 50 µg/kg of diet. At 11 weeks of age, the rats were started on the various experimental rations.

TABLE 1

Composition of basal diet

	% of diet
Sucrose	65
Soybean protein [1]	20
Salts [2]	5
Cottonseed oil [3]	10
Vitamins [4]	*mg/100 g diet*
Thiamine·HCl	1.6
Pyridoxine·HCl	1.6
Riboflavin	1.6
Ca pantothenate	4.0
Nicotinic acid amide	5.0
Folic acid	0.5
Inositol (meso)	10.0
dl-α-Tocopheryl acetate	10.0
Menadione	1.0
	IU/100 g diet
Vitamin A	2,500
Vitamin D_3	600

[1] Promine D, Central Soya Company, Chicago (1.0% methionine).
[2] Hegsted, D. M., R. C. Mills, C. A. Elvehjem and E. B. Hart 1941 J. Biol. Chem., 138: 459.
[3] Weston Oil, Weston Sales Company, Fullerton, Calif.
[4] Vitamin B_{12} (50 µg/kg of diet) was added to control diets.

Females were fed the experimental ration for 4 weeks prior to breeding. Male animals, fed a commercial ration, were housed separately until the time of breeding when they were placed with the females, one male per cage. Vaginal smears were checked daily for the presence of sperm. Females which were found to be positive were moved to single cages and fed the experimental diets throughout gestation.

The neonatal offspring from the eight groups of animals, consisting of six females each, were used. Control animals which were supplemented with vitamin B_{12} during the growth period were placed on diets 1 and 2, whereas vitamin B_{12}-deficient animals were given diets 3 through 8 (table 2). The weights of the litters, the number of embryos per litter, and the number of hydrocephalic animals were recorded. Various litters were chosen consecutively for the isolation of brain phospholipids, or for the determination of the incorporation of ^{14}C-methyl-labeled methionine into various chemical fractions of the liver and brain. Hydrocephalic animals were excluded from the litters on which chemical analyses were performed, with the exception of litters containing all hydrocephalic individuals which were analyzed separately.

The results presented in the tables represent the mean values obtained from individual samples within each dietary treatment. The data in tables 4 and 5 were treated statistically using an analysis of variance. An *F* value was computed to determine differences between dietary treat-

TABLE 2

Experimental diets

Diet no.	Addition to basal diet
1	0.18% choline bitartrate + 0.32% DL-methionine + vitamin B_{12} [1]
2	vitamin B_{12} [1]
3	0.18% choline bitartrate + 0.32% DL-methionine
4	0.18% choline bitartrate
5	0.32% DL-methionine
6	0.64% DL-methionine
7	0.96% DL-methionine
8	None

[1] Vitamin B_{12} added at the rate of 50 µg/kg diet.

[4] Charles River Laboratories, Inc., Wilmington, Mass.

ments. In tables 4 and 6 each dietary group was then compared with the vitamin B_{12}-supplemented control group.

Brain phospholipids. The analysis of phospholipids was conducted on neonatal rats whose mothers were fed experimental diets 1, 3 and 5 (table 2). Each analysis was performed on a sample consisting of brains from two littermates, and each sample was run in triplicate. In this way the phospholipids from a total number of 32 litters were examined. The lipids were extracted by the method of Folch et al. (11), and analyzed quantitatively by the procedure outlined by DeBolner et al. (12). The extract was evaporated under nitrogen and adjusted to a constant volume so that 4 µg of phosphorus would be present in 25 µl of lipid extract.

Chromatoplates were prepared using silica-gel G according to the method of Stahl (13). The sample was applied, and the plates were developed in the alkaline solvent mixture described by Müldner et al. (14). After drying, detection of spots was carried out with iodine vapors. The silica containing each spot was removed and the phospholipids eluted twice using the method employed by Marinetti et al. (15). The eluted phospholipids were ashed and analyzed for phosphorus according to the method of Chen et al. (16).

Incorporation of L-*methionine-methyl-*^{14}C. Six individual embryos from a litter received subcutaneous injections of L-methionine-methyl-^{14}C (17.0 mCi/mmole) at the rate of 0.1 µCi/g embryo. Litters were injected with radioisotope within 4 hours of birth and only viable active embryos were chosen for biochemical determinations. Care was taken that the subcutaneous injection of isotope did not escape from the needle puncture hole. An "incorporation time" of 1 hour showed maximum uptake of methionine into serum proteins; therefore this time period was used throughout these experiments. Embryos were exsanguinated after 1 hour by decapitation at the atlanto-occipital joint. Livers and brains were removed; the different organs from six animals of one litter were placed in separate beakers containing cold 0.25 M sucrose. The total weight of the liver and brain was determined. A 20% homogenate was obtained, and duplicate samples were then separated into acid-soluble, lipid, proteolipid, protein, and nucleic acid components using a modification of the extraction techniques of Schneider (17).

The chloroform-methanol extract outlined by Schneider was separated into two components by adding distilled water to the lipid solvent. The buffy coat layer (proteolipid) which formed at the aqueous methanol-chloroform interphase was removed from the chloroform-soluble lipid by differential freezing. The amount of lipid was determined by weight after evaporating the chloroform with nitrogen. The Folin-Ciocalteu method was used to quantitate the amount of protein and proteolipid (18). RNA was determined using orcinol reagent, and DNA was determined by the diphenylamine reaction as outlined by Schneider (19). Equal volumes of each fraction were used for the chemical and radioactive determinations. Proteinaceous materials were dissolved using hydroxide of Hyamine.[4] Dissolved fractions were added to scintillation fluid (20), stored in the dark for 24 hours to prevent chemiluminescence, and counted with a liquid scintillation counter.[5] Efficiency and the degree of quenching were determined by the external standard ratio method.

RESULTS

The incidence of hydrocephalus was highest in animals whose dams were fed a vitamin B_{12}- and choline-deficient diet and supplemented with varying quantities of methionine (diets 5, 6 and 7, table 3). These results are similar to those obtained in previous experiments (8). When methionine was added in amounts equivalent to choline in methyl groups (diet 5), hydrocephalus was not prevented. Twice the number of moles of methionine did not reduce the incidence; however, depression was observed when the level of methionine was increased to 6.3 mmoles/100 g diet. When comparisons of brain, liver or total embryo weights were made, a statistical difference was noted; however, within groups comparisons showed that these dif-

[4] p(Diisobutyl-cresoxyethoxyethyl) dimethylbenzyl ammonium hydroxide, Packard Instrument Company, Downers Grove, Ill.

[5] Beckman model DPM 100, Beckman Instruments, Inc., Fullerton, Calif.

TABLE 3
Incidence of hydrocephalus

Diet no.	Supplement to basal diet	No. of litters	Litters hydrocephalic	No. of embryos	Embryos hydrocephalic
	mmoles/100 g diet		%		%
1	Choline, 0.7 + methionine, 2.1 + vitamin B_{12}[1]	23	0	143	0
2	Vitamin B_{12}[1]	33	0	196	0
3	Choline, 0.7 + methionine, 2.1	22	0	160	0
4	Choline, 0.7	13	0	95	0
5	Methionine, 2.1	17	24	132	7
6	Methionine, 4.2	14	36	110	14
7	Methionine, 8.3	13	7	107	1
8	None	13	0	80	0

[1] Vitamin B_{12} added at the rate of 50 μg/kg diet.

ferences were not the result of vitamin B_{12} deficiency (table 4).

Four different phospholipid components were isolated from the brains of neonatal rats. The identification of the phospholipids was based upon R_f values (12) and the use of various spot tests as outlined by Skidmore and Entenman (21). The phospholipids identified were phosphatidyl serine, sphingomyelin, lecithin and phosphatidyl ethanolamine (fig. 1). With the system employed phosphatidyl inositol should migrate with phosphatidyl serine; however, it could not be detected in this experiment by means of spot tests. The results of the quantitative measurements of the four phospholipids indicate that there was no significant difference between any of the experimental groups; however, some variation among the triplicate samples was encountered (table 5).

The results of the isolation and quantitation of the various biochemical constituents from embryonic livers and brains are shown in table 6. The results represent the means obtained from six litters containing six neonates each. Each litter was obtained from a separate female rat on the dietary treatment. Differences between duplicate samples were found to be relatively small; however, very large differences were found between individual litters. Statistically significant differences were found between some mean values, but, when comparisons were made between dietary treatments these differences became nonsignificant. There were insufficient data obtained from completely hydrocephalic litters for statistical evaluation, but it was obvious by inspection that no differences existed. Fluctuations observed in the incorporation of methionine or its methyl group into the various chemical constituents were related to endogenous differences in neonatal metabolism, and could not be correlated with a deficiency of vitamin B_{12} or methyl groups during embryonic development.

DISCUSSION

The dietary experiments indicated that a higher incidence of congenital abnormalities is produced when choline- and vitamin B_{12}-deficient diets are supplemented with methionine. In our experience it has not been unusual to find that dams fed diets deficient in vitamin B_{12}, but supplemented with choline, give birth to relatively few anomalous young, although the mothers have low liver levels of vitamin B_{12}. It is, therefore, not surprising that no congenital anomalies were observed in the groups fed diets 3 and 4. The addition of 4.2 mmoles of DL-methionine/100 g diet (diet 6, table 3) did not reduce the incidence of congenital anomalies although the dams received twice the number of methyl groups furnished by choline (diet 4). The highest levels of methionine (diet 7) also failed to totally prevent hydrocephalus. These results are in contradistinction to the sparing action that methionine exerts in the choline-deficient hemorrhagic kidney syndrome of weanling rats (22). The dichotomy of the methionine action in these two models could be explained

TABLE 4

Weight comparisons

Diet no.	Supplement to basal diet	Avg embryo wt	Brain wt per six embryos	Liver wt per six embryos
	mmoles/100 g diet	*g*	*g*	*g*
1	Choline, 0.7+ methionine, 2.1+ vitamin B_{12}[1]	6.3	1.5	1.9
2	Vitamin B_{12}[1]	5.4	1.4	1.3
3	Choline, 0.7+ methionine, 2.1	6.0	1.4	1.6
4	Choline, 0.7	5.0	1.2	1.2
5	Methionine, 2.1	5.0	1.3	1.4
6	Methionine, 4.2	5.7	1.4	1.5
7	Methionine, 6.3	6.0	1.5	1.5
8	None	5.5	1.4	1.6
Significance of dietary group comparisons		0.05	0.01	0.05
Significance of pairwise comparisons		nsc[2]	nsc	5 versus 1 (0.01) 4 versus 1 (0.05) 2 versus 1 (0.01)

[1] Vitamin B_{12} added at the rate of 50 μg/kg diet.
[2] nsc = no significant comparison.

TABLE 5

Analysis of brain phospholipids

Phospholipids	Dietary treatment		
	1	3	5
	μg P/g fresh tissue		
Phosphatidyl serine	467	391	310
Phosphatidyl ethanolamine	753	639	704
Lecithin	1148	944	926
Sphingomyelin	327	266	338
Significance	ns[1]	ns	ns

[1] ns = not significant.

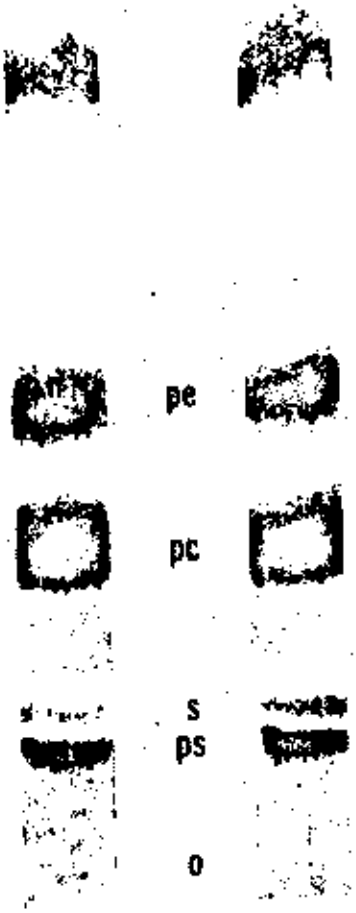

Fig. 1 Typical thin-layer chromatograms of phospholipids isolated from fetal brains. o = origin, ps = phosphatidyl serine, s = sphingomyelin, pc = phosphatidyl choline or lecithin, and pe = phosphatidyl ethanolamine.

if a minimal dietary level of methyl groups was necessary for embryonic development and fetal growth. Since methionine is an essential amino acid, it would serve a dual function in protein synthesis and methyl neogenesis.

Inhibition of phospholipid synthesis plays a direct role in the pathogenesis of choline-deficiency fatty liver and is of questionable significance in the hemorrhagic kidney syndrome of weanling rats. The role phospholipids play in the pathogenesis of nutritionally induced neural developmental anomalies was investigated by isolating and quantitating the various phospholipid components. No differences in the

TABLE 6

Biochemical analysis and incorporation of L-methionine-methyl-^{14}C

Diet no.	Supplement to basal diet	DNA: Avg mg DNA/animal	DNA: mg DNA/g wet wt	DNA: dpm/mg DNA	RNA: mg RNA/g wet wt	RNA: dpm/mg RNA	Protein: mg protein/g wet wt	Protein: dpm/mg protein	Lipid: mg lipid/g wet wt	Lipid: dpm/mg lipid	Proteolipid: mg protein/g wet wt	Proteolipid: dpm/mg protein	Acid soluble: dpm/g wet wt
	mmoles/100 g diet						Liver						
1	Choline, 0.7+ methionine, 2.1+ vitamin B_{12}[1]	0.62	1.9	512	104	42	79	688	18.1	3998	26.2	1071	138,532
2	Vitamin B_{12}[2]	0.57	2.7	308	123	64	81	1133	22.8	5313	29.7	1428	225,207
3	Choline, 0.7+ methionine, 2.1	0.60	2.4	742	88	76	78	1582	22.6	11136	38.7	1741	97,936
4	Choline, 0.7	0.58	3.1	608	121	107	84	1114	17.2	8325	49.8	812	125,196
5	Methionine, 2.1	0.51	2.5	794	115	68	89	1906	25.0	5548	26.2	1953	121,654
6	Methionine, 4.2	0.49	2.1	661	96	76	82	1436	18.9	8594	30.5	1339	117,598
7	Methionine, 6.3	0.38	1.5	723	121	67	83	1097	21.0	5964	33.5	1539	120,814
8	None	0.66	2.3	235	95	77	70	853	26.1	5090	29.3	1271	119,035
Significance between diets		ns[3]	ns	ns	ns	ns	ns	0.05	ns	0.01	ns	ns	ns
Significance of pairwise comparisons		nsc[4]	nsc	nsc	nsc	nsc	nsc	5 versus 1 (0.05)	nsc	5 versus 3 (0.10) 2 versus 3 (0.10) 6 versus 3 (0.10) 1 versus 3 (0.05)	nsc	nsc	nsc
							Brain						
1	Choline, 0.7+ methionine, 2.1+ vitamin B_{12}[1]	0.26	1.0	296	32.0	37	48.0	366	23.0	106	7.9	343	64,092
2	Vitamin B_{12}[2]	0.56	2.6	129	35.8	91	51.0	514	21.9	113	7.4	479	61,360
3	Choline, 0.7+ methionine, 2.1	0.32	1.4	528	30.1	110	37.0	1207	21.0	161	12.3	826	67,060
4	Choline, 0.7	0.40	1.8	690	27.0	221	43.0	638	21.9	155	14.5	614	63,325
5	Methionine, 2.1	0.39	1.9	708	35.1	137	50.0	1354	22.4	184	9.2	1050	87,874
6	Methionine, 4.2	0.30	1.3	514	27.2	95	47.0	706	21.2	150	9.5	482	67,294
7	Methionine, 6.3	0.40	1.7	536	33.5	101	49.0	570	21.9	143	6.8	1314	59,013
8	None	0.37	1.5	247	37.8	67	43.0	453	21.7	97	8.3	430	85,213
Significance between diets		ns	ns	ns	0.01	0.10	0.10	0.01	ns	ns	0.05	ns	ns
Significance of pairwise comparisons		nsc	nsc	nsc	nsc	nsc	nsc	5 versus 1 (0.10)	nsc	nsc	nsc	nsc	nsc

[1] Vitamin B_{12} (50 μg/kg diet) was fed throughout growth period.
[2] Animals raised on a vitamin B_{12}-deficient diet throughout growth period and then supplemented with vitamin [illegible]
[3] ns = not significant.
[4] nsc = no significant comparison.

brain phospholipid patterns could be detected between neonates whose dams were raised on vitamin B_{12}-supplemented or deficient diets, or between offspring whose mothers were fed diets severely deficient in lipotropic agents. Likewise, the incorporation of methyl groups from methionine into brain and liver lipids was not significantly different. These results indicated that the congenital neural abnormalities induced by vitamin B_{12} deficiency cannot be attributed to major alterations in the synthesis of phospholipids. Total lipid analysis of neonatal livers showed that the animals whose dams were fed diets deficient in lipotropic substances did not have increased amounts of liver fat although the livers from their mothers had microscopic evidence of severe fatty metamorphosis. The incorporation of L-methionine-methyl-^{14}C into various chemical components indicated that there were no major shifts in the methyl requirements of the various tissue constituents. The constant specific activities found in the various biochemical substances might be attributed to the fact that dams are furnishing methyl groups for embryonic growth at the expense of regulating their own methyl homeostasis.

The total amounts of DNA and RNA per gram tissue were not found to be changed by a deficiency of vitamin B_{12}. These results differ from those previously reported by other workers (5, 23) who found a decrease in the amount of RNA and an increased amount of DNA within the brain. As explained by O'Dell and Bruemmer (23) the differences in the content of nucleic acids previously reported might result because deficient offspring failed to feed as well as control animals. Care was taken in our experiments to ensure that newborn animals did not nurse; this was verified by examining the stomach contents at necropsy. It should be noted, however, that the tissues in our experiment were obtained within 5 hours of birth, whereas the tissues in the previous experiments were obtained from 1-day-old neonates. Since maternal nutrition has been severed there is sufficient time difference between the two experiments to allow major metabolic alterations to occur.

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A FURTHER STUDY OF THE APPARENT SYNTHESIS OF VITAMIN B_{12} BY MAMMARY CANCERS OF MICE

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In a previous paper[1] evidence was reported which indicated that mice bearing spontaneous mammary cancers synthesized vitamin B_{12}. Nontumorous animals, on the contrary, did not seem to form this vitamin in amounts sufficient for normal requirements. This finding of apparently greater synthesizing capacity in the cancerous animals ran counter to a prevailing belief, which is that cancers may differ from normal tissues by lacking some particular power of synthesis. Furthermore, the finding of the apparent ability of mammary cancers to synthesize vitamin B_{12} (in contrast to the lack of such ability in the normal animal) provided a rational basis for the elaboration of drugs which would poison the tumor without, at the same time, harming the host animal.[1, 2, 3] Such drugs have actually been produced.[4, 5] Consequently, because of the importance attributable to the finding about vitamin B_{12} synthesis in the cancerous mice, it became advisable to seek supporting evidence for such a property of the tumors.

The preceding evidence for the synthesis of vitamin B_{12} by mice bearing spontaneous mammary tumors was obtained by animal assay. The young of cancerous mothers maintained on the B_{12}-deficient diet grew normally, indicating that the mothers had acquired and transferred to them enough vitamin to meet their needs for normal growth. This excess vitamin B_{12} probably was synthesized by the cancer of the mother, or at least by the mother in response to her cancer, because she was subsisting on a B_{12}-deficient ration. The evidence to be presented in this paper was obtained by microbiological assay. It has been found that tumorous mice maintained on a vitamin B_{12}-deficient diet contained more vitamin B_{12} than did similarly treated nontumorous mice. Thus, by direct assay of the tissues by a microbiological procedure, the cancerous animals were found to contain more of the vitamin than the noncancerous controls. This excess probably arose from synthesis by the tumor.

Other explanations for the data can be proposed. One of these is that the tumorous animals stored the vitamin more than did the noncancerous controls. One may say that, although the diet contained no demonstrable vitamin B_{12}, it may have carried traces. These traces may have been filtered out and stored by the cancerous mouse more efficiently than by the control animal. No adequate evidence to disprove such an explanation was found, but some of the data were less readily understood on such a basis than on the idea of synthesis of the vitamin by the tumor.

EXPERIMENTAL

Diets and Animals.—Except where otherwise noted, the B_{12}-deficient ration of Zucker and Zucker was used.[6] This ration was composed principally of a commercially available flour made from cottonseed meal and known as "Proflo." It was found quite essential to use Proflo made by the old process. The product of the

same name made by the new process failed consistently to produce deficient animals. Some experiments were carried out with the highly purified ration of Hartman *et al.*, in which alcohol-extracted casein constituted 45 per cent of the mixture.[7]

All the mice were of the Swiss strain maintained in this institute. These were not the Webster Swiss, which is the strain offered for sale by many dealers. As the females of the Swiss strain grew old, a rather large percentage of them developed mammary cancers. For the present experiments a large number of these old females were kept on stock rations and examined weekly. As soon as a mouse showed a palpable mammary tumor, she was placed on the deficient diet and fed in this manner until the cancer had grown to a large size. The animals were caged individually in screen-bottomed boxes and provided with food and water.

Selection of a Method for Microbiological Assay.—The chrysomonad method was the only one found satisfactory for this investigation. Most of the determinations were carried out according to the directions of Hutner *et al.* and Barber *et al.*[8] It was found essential to eliminate completely from the solution to be assayed any trace of turbidity due to fat or to suspended particles which may have passed through the paper when the unknown was filtered. This was accomplished by centrifugation at 14,000 rpm. If this was not done, fictitiously high values were frequently found. When the revised procedure was published by Ford,[9] it was adopted for the remainder of the investigation because it reduced the dangers arising from tiny particles or from emulsified fat. Before this was done, however, many of the samples which had been analyzed by the older procedure were reexamined by Ford's modified method. The results on any sample tested by both procedures agreed well.

The reason for the selection of the chrysomonad method was its greater specificity. This organism does not respond to pseudo-vitamins B_{12} which are found in the intestines of animals but which show no vitamin B_{12} potency for mammals and birds. Because whole mice were to be assayed in the present work, this was an important consideration. In the beginning, attempts were made to use the coli-mutant method.[10] To test its suitability, normal Swiss mice were fed the deficient ration for 3 days, and individual animals were taken for assay. The whole mouse was ground in a Waring Blendor with 10 volumes of water, and the homogenized sample was digested, autoclaved, and assayed (cf. Scheid *et al.*[11]). Values obtained on such samples by the coli method were usually much higher than by the chrysomonad method (see Table 1). Furthermore, removal of stomach and intestines from the mouse before assaying markedly lowered the value found by the coli

TABLE 1

COMPARISON OF B_{12} CONTENTS OF NORMAL MICE, AS DETERMINED BY THE CHRYSOMONAD AND COLI METHODS

	B_{12} Content (Milligamma per Gram)			B_{12} Content (Milligamma per Gram)	
	Coli-Mutant	Chrysomonad		Coli-Mutant	Chrysomonad
Mouse:			*Mouse minus stomach and intestines:*		
A1	26	12.7	A23	17	12.4
A2	20	6.4	A24	7	10.6
A3	65	13.0	A25	10	9.8
A4	21	7.8	A27	10	14.5
A6	86	9.0	A28	7	10.8

method but did not materially change that obtained with the chrysomonad procedure. These data also are shown in Table 1. This latter finding tended to suggest that pseudo-vitamins B_{12} accounted for much of the extra vitamin found with the coli method. Because the coli values had been corrected for methionine by separate assay in the way recommended,[19] the high results obtained by this method could not be attributed to methionine. These preliminary studies were taken as evidence that the chrysomonad method was the one which should be used.

Many experiments were performed to test the reproducibility of the values obtained on any given sample by the chrysomonad procedure. These indicated that the method was satisfactory from this standpoint. For example, mouse A91 was found to contain 10.7, 11.0, 11.0, 8.7, and 11.0 milligamma of vitamin B_{12} per gram in five separate assays. Similarly, recovery of vitamin B_{12} added to normal mice was tested and found to be satisfactory, in that 80–100 per cent of added vitamin could be recovered. The data of Table 1 will also indicate the variation among individuals in a group and will show that most of the animals had about the same content of vitamin B_{12}. This was considered of importance in the evaluation of subsequent results. In all the analyses subsequently described in this paper, the stomach and intestines were removed from the mice before they were ground for the assay.

Vitamin B_{12} Content of Nontumorous Mice Fed the Deficient Diet.—Groups of three to eight adult female Swiss mice were fed the deficient ration in the manner indicated above, and, after various intervals of time, each animal was assayed. The data of Table 2 show that the average B_{12} content was greater if the mice had been receiving the deficient ration for a short time but that, after they had eaten it for 2.5 weeks, no further significant change in B_{12} content was detectable.

Similarly, the effect of age of the mouse at the start of the feeding of the deficient diet was studied. This was important, because most of the tumorous mice would be old and of varying age. It was found that the animals approached the same content of vitamin B_{12} when they were given the deficient ration, regardless of whether they were 2 months of age or 9 months of age when they were started. In other words, any normal adult mouse approached a vitamin B_{12} content of about 9 milligamma per gram when it was fed this deficient diet for several weeks.

Vitamin B_{12} Content of Mice Bearing Spontaneous Mammary Cancers when Fed the Deficient Ration.—Adult Swiss mice bearing small spontaneous mammary tumors were fed the deficient ration until the cancers were large. The time required for this was usually about a month, but in some instances it was considerably longer. They were then analyzed in the way described. At the same time, normal Swiss mice of the same age were tested in the same way. The results, shown in Table 3, indicate that the cancerous mice contained about twice as much of this vitamin as did the controls.

Vitamin B_{12} Content of Normal, Pregnant Mice Fed the Deficient Diet.—Because many differences of cancerous mice from normals have, in the past, been shown to be due only to the resemblance of the cancer to embryonic tissue, it was considered advisable to determine whether this same criticism might be leveled against the difference with respect to vitamin B_{12}. A group of twelve normal Swiss mice in the first week of pregnancy was fed the deficient ration until the day of parturition or until a few days before this time. Each mouse was then analyzed for vitamin B_{12}.

The average content was 9.5 milligamma per gram. This value, when compared to 9.4 milligamma per gram for eight nonpregnant controls, showed that there was no evidence for synthesis of the vitamin by the embryos.

Vitamin B_{12} Content of Spontaneous Mammary Cancers in Comparison to the Vitamin B_{12} Content of the Host Mice from Which They Came.—When a cancer-bearing mouse was fed the deficient diet for several weeks, and its tumor was excised and assayed at the same time as the remainder of the carcass, the content in the carcass was usually found to be somewhat greater than that in the tumor. Thus in mouse 275 the carcass contained 23 milligamma per gram, and the tumor 10 milligamma per gram. The corresponding average values from six such animals were 30 and 15.

Destruction of Vitamin B_{12} by Excised Spontaneous Mammary Cancers.—One reason for the lower B_{12}-content of the cancers in comparison to the remainder of the carcass was found to be that the cancer, when minced in the presence of the vitamin, destroyed some of it. Presumably, a similar destruction of endogenous vitamin occurred when the cancer was ground for assay. In a typical experiment, mouse A84 was maintained on the deficient ration, and her cancer, weighing 2.6 gm., was excised. Half of it was minced with scissors in 13 cc. of water and then ground with sand. The mixture was incubated for 1 hour at 37° C. The suspension was then autoclaved, and the clear supernatant liquid was assayed. Two and one-half milligamma of vitamin B_{12} per gram of fresh tissue were found. The other half of the cancer was mixed with 20 milligamma of vitamin B_{12} per gram of fresh tissue before mincing, but other operations on it were the same. It was found to contain 13 milligamma per gram, whereas it should have contained 22.5. Clearly, some of the added vitamin had not been recovered.

TABLE 2

AVERAGE B_{12} CONTENT OF NORMAL MICE FED THE DEFICIENT RATION FOR VARYING PERIODS OF TIME

Period on Deficient Ration (Weeks)	No. of Mice	Average B_{12} Content (Milligamma per Gram)
0	3	24.0
0.5	6	12.5
2.5	8	8.9
5.0	6	9.0
Greater than 10	6	9.5

TABLE 3

VITAMIN B_{12} CONTENT OF DEFICIENT MICE BEARING SPONTANEOUS MAMMARY CANCERS, INCLUDING DATA ON THE EFFECT OF EXCLUSION OF THE TUMOR FROM THE SAMPLE

Description of Mice	No. of Animals	Average B_{12} Content (Milligamma per Gram)
Noncancerous	24	9.3
Cancerous	13	17.2
Cancerous, minus tumor	13	28.7

Each tumor examined in this fashion was not found to destroy the same amount of vitamin B_{12}. Some destroyed very little (see Table 4). This variation among individual cancers may explain why the B_{12} content of a few cancer-bearing individuals was found to be much less than the average for such mice. If a cancer was very active in destruction of the vitamin, the value for B_{12} content found after homogenization of the mouse plus cancer would be unusually low.

Vitamin B_{12} Content of Cancerous Mice Analyzed without Their Cancers.—The experiment on the B_{12} content of cancerous mice maintained on the deficient ration was repeated, except that the cancer was removed from each mouse before it was

ground for analysis. The data in Table 3 show that under these conditions the animals were found to contain considerably more of the vitamin than if their tumors had not been removed. The results of this experiment indicated that the cancer tissue was destroying some of the vitamin B_{12} when carcass plus cancer were ground together before the analysis. Nevertheless, even with this technique of discarding the cancer before grinding, the B_{12} content of a cancerous mouse might be as high as 55 milligamma per gram, while others might show only 7 milligamma per gram. It may be that various strains of cancer differ quantitatively in ability to synthesize vitamin B_{12} as well as to destroy it.

EXPERIENCES WITH TRANSPLANTED MAMMARY CANCERS

a) Vitamin B_{12} Synthesis (as Suggested by Content) of Mice Bearing Cancer Strain W137.—As explained more fully elsewhere,[8] transplanted mammary cancer strain W137 was obtained from one of the Swiss mice of the present study and was transplanted in Swiss mice. The experiments on B_{12} synthesis were performed with the fourth to eighth passages of this strain. This strain was of interest because it was the most susceptible to antimetabolites of dimethyldiaminobenzene and should, therefore, show evidence of B_{12} synthesis if the general working hypothesis[1, 9] has

TABLE 4

DESTRUCTION OF VITAMIN B_{12} BY SPONTANEOUS CANCERS IN VITRO*

Mouse	B_{12} Content (Milligamma per Gram) Tumor	Tumor and B_{12}	Percentage Destruction
A84	2.5	13.0	50
146	19.0	37.6	7
275	10.0	21.0	45
277	21.0	35.0	30

* The tumor tissue was incubated with 20 milligamma of vitamin B_{12} per gram of fresh tissue for one hour at 37°.

validity. Three mice bearing established transplants of W137 were fed the deficient ration for one week. The tumors were discarded, and the carcasses were analyzed for vitamin B_{12}. The average value was 30 milligamma per gram, which was much more than in the controls (cf. Table 1).

A second set of experiments was performed in this way, except that the Proflo ration was replaced by the B_{12}-deficient diet of Hartman *et al.*[7] This was a semisynthetic diet rich in alcohol-extracted casein. The nontumorous control mice had an average B_{12} content of 18 milligamma per gram. The mice bearing transplanted cancer strain W137 contained 24 milligamma per gram. Evidently this ration did not render mice so deficient in vitamin B_{12} as did the Proflo diet and therefore did not magnify so much the difference in the two kinds of mice.

b) Synthesis versus Storage in Strain W137.—One might contend that by using well-established cancer transplants one had given opportunity to the host plus cancer to store extra vitamin B_{12} before the deficient ration was fed. The resultant greater content of the vitamin in the animal thus might represent not synthesis but, rather, merely more tenacious storage. This point was tested in the following way.

Swiss mice were fed the Proflo-containing deficient diet for two weeks. Half of them were then implanted with strain W137, and the others were maintained as controls. Both groups were continued on the deficient ration, and, when the tu-

mors were well developed (3–4 weeks), analyses were performed. The average B_{12} content of the controls was 10 milligamma per gram, and of the cancerous mice, 15.5 milligamma per gram. This result tended to suggest that the tumor strain W137 transplanted into animals depleted in vitamin B_{12} led to accumulation of this vitamin in the carcass, presumably by synthesis. However, a garnering and storage of traces of the vitamin which may have been in the diet cannot be entirely excluded as an explanation.

e) Failure of Transplanted Strain W113 To Give Any Evidence for Synthesis.—In the preceding paper on the evidence for B_{12} synthesis by spontaneous mammary cancers,[1] the failure to find any such synthesis with a transplanted strain was recorded.[12] In the present study, another strain of transplanted mammary cancer of Swiss mice has likewise been found to give no evidence for synthesis of vitamin B_{12}. Clearly, then, some transplanted strains do and some do not give evidence for synthesis.

Swiss mice were implanted with strain W113,[4] and 1–2 weeks later, when the tumors were established, the animals were fed the deficient diet. Two weeks later (when the tumors were large) the animals were killed and their tumors removed and discarded. Analyses of the carcasses of six of these mice showed them to contain, on the average, 5 milligamma of vitamin B_{12} per gram. Therefore, the vitamin content was somewhat less than in nontumorous controls (cf. Table 1).

d) Active Destruction of Vitamin B_{12} by Transplanted Tumor Strain W113.—The strain which failed to give evidence for synthesis was found to destroy vitamin B_{12} actively. Eight mice implanted with strain W113 were treated exactly as described in the preceding section, except that the tumors were not removed from the animals before the analyses were performed. The average B_{12} content of these mice was 2.1 milligamma per gram. Because the nontumorous controls had more than three times this amount, it was clear that the cancer had destroyed much of the B_{12} in the sample when the whole mouse was ground. This experience with strain W113 gave the most clear-cut example of B_{12} destruction which has been seen in this work and led to the trials on B_{12} destruction by spontaneous cancers which were described earlier in this paper. In the case of the spontaneous cancers, the destruction of the vitamin was not found to be as extensive as with this transplanted strain.

Direct proof of destruction of vitamin B_{12} by the excised cancer was obtained in the way described for the similar experiments with the excised spontaneous tumors. With strain W113, 1 gm. of minced cancer tissue was incubated 1 hour at 37° with 10 milligamma of B_{12}. Analysis showed that only 2.9 milligamma of the vitamin remained. Since the tissue without added B_{12} gave a value of 1.2 milligamma per gram, 74 per cent destruction of added vitamin had taken place.

Four strains of transplanted mammary cancers were examined in the way just described for their abilities to synthesize vitamin B_{12} while growing in Swiss mice and to destroy it when incubated in vitro. Evidence for synthesis has been obtained for three of the strains, and evidence for destruction (at least to some extent) with all four. The most active destroyer was W113, just described, for which no evidence for synthesis was found.

DISCUSSION

Although the data of this paper serve as additional evidence for the idea that spontaneous mammary cancers of mice synthesize vitamin B_{12}, they do not establish this idea as a fact beyond question. It has not been possible to eliminate the alternative that the tumorous animals trap and store traces of this vitamin which may be present in the deficient ration. The difference from normal animals would, if this were the true explanation, then reside in a greater capacity of the cancerous animals for trapping and storage of vitamin B_{12}. However, the present evidence obtained with microbiological assays corroborates that found previously by animal assay in showing that the cancer-bearing mice either synthesize or store more B_{12} than do normal controls.

The destruction of vitamin B_{12} by certain mammary cancers is noteworthy. Some enzymic system was possibly responsible for this destruction, since the activity was lost by heating the tissues. Prior to this finding, no evidence for a B_{12}-destroying system has come to our attention. To be sure, there are preparations from normal gastric juice and from other sources which combine with, and thus inactivate the microbiological activity of, this vitamin. However, this activity may be regained by heating the reaction mixture. In the present case, however, the activity could not be regenerated by heating and may represent true destruction of the molecule.

Some may wonder whether the destroying system is anything except the B_{12}-synthesizing system acting in reverse. This may well prove to be the case. However, present evidence does not permit any conclusions on this point.

The variations among individual tumorous mice both in the amount of apparent synthesis of vitamin B_{12} and in B_{12}-destroying capacity were striking. Thus, although control animals maintained on the deficient diet each had approximately the same B_{12} content, the variation among cancerous mice was rather large. Some showed five times the average nontumorous content; one had less than the average of the controls. The majority of the cancerous individuals did, however, cluster around a B_{12} content which was twice that of the controls. This failure of a few of the cancerous individuals to show evidence for synthesis was found not only by the methods of the present study. Careful examination of the records of the older investigation[1] showed that there, too, was an individual which gave no evidence for synthesis. Clearly, then, there are some mice which, when invaded by the cancer, do not show evidence for B_{12} formation. In the case in which the cancer was examined in a transplanted condition (i.e., strain W113), failure to find any evidence for synthesis was associated with the greatest capacity for destruction of the vitamin. It remains to be determined whether causal relationship existed.

SUMMARY

A microbiological method for the determination of vitamin B_{12}-content of mice was sought. The coli-mutant method was found to be unsatisfactory in comparison to the chrysomonad method. When the chrysomonad method was employed, analyses of nontumorous mice fed a B_{12}-deficient ration showed them to contain on the average, 9 milligamma of the vitamin per gram of live weight. The correspond-

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ing value for similar mice bearing spontaneous mammary cancers was 17 milli-grams. The extra B_{12} was thought to have arisen from synthesis by the tumor. Other explanations were also considered. A transplanted strain of these cancers was found to give similar evidence for vitamin B_{12} synthesis. A second trans-planted strain showed no evidence for such synthesis but, instead, was demon-strated to bring about active destruction of the vitamin in vitro. Several individ-ual spontaneous mammary cancers were likewise shown to destroy this vitamin, but not so actively as did the one transplanted strain. This destruction was shown to be different from mere combination of a protein with the vitamin, such as is known to occur in gastric juice.

* With the technical assistance of V. Armbrust and G. Schaffner.

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PERMANENT CURE OF SOME SPONTANEOUS MAMMARY CANCERS OF MICE WITH ANALOGS OF 1,2-DIMETHYL-4,5-DIAMINOBENZENE

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Abstract—A mixture of 1,2-dimethyl-4-(*p*-carboxyphenylazo)-5-hydroxybenzene (CPA) and 1,2-dichloro-4-benzenesulfonamido-5-nitrobenzene (DCBN), when fed to white mice of the SPFS strain bearing spontaneous mammary cancers, cured permanently about one-third of the animals. In others, transient cures were observed, but the cancers eventually returned. The maximal number of cures was obtained with 6 g CPA and 0·5 g DCBN per kg of ration. Additional amounts did not increase the percentage of cures. A related compound, 1,2-dimethyl-4,5-*bis*(benzenesulfonamido)benzene (DMDB), also cured some cancers but seemed less potent than the combination of CPA and DCBN. Some other relatives were apparently inactive. In C3H mice, bearing spontaneous mammary cancers, CPA plus DCBN caused transient regressions of the neoplasms but did not result in permanent cures. Several substances such as aminopterin, 5-fluorouracil and 5-fluorodeoxycytidine, which inhibit the growth of certain transplanted cancers of mice, did not exert any detectable effect on these *spontaneous* cancers. The effective compounds were conceived on the basis of the demonstration that the spontaneous cancers differed from normal host tissues in that they synthesized vitamin B_{12}. The new compounds are members of a series of antimetabolites which have been shown to inhibit the biosynthesis of this vitamin in microorganisms. However, there was no direct proof that the effect on the cancers was the result of the relationship to vitamin B_{12} synthesis. The compounds exerted no detectable harmful effects on the host mice at the doses used to bring about cure of the cancers. This selective toxicity was predicted from knowledge of the relationship of the analogs to the biosynthesis of vitamin B_{12} and riboflavin.

PRECEDING papers have presented evidence indicating that certain spontaneous mammary cancers of mice differed from normal mouse tissues in that the cancers synthesized vitamin B_{12}.[1, 2] This metabolic difference allowed the prediction and realization of antimetabolites which were poisonous to the cancers but harmless to the host mice.[3, 4] These antimetabolites were structural analogs of 1,2-dimethyl-4,5-diaminobenzene, a compound that has been shown to serve as a precursor for the biosynthesis of vitamin B_{12} in microbial species.[5, 6]

Although the antimetabolites described earlier were able to harm the cancers selectively, they were not able to cure spontaneous mammary cancers permanently. All that was observed was transient regression in some of the tumors and transient obliteration of a few of them. In about half the animals no effect on the growth of the tumors was observed.[3, 4] In those that were affected, the effect was transient. After

* With the technical assistance of C. Maugé. A summary of this paper was read at the First International Pharmacological Meeting, Stockholm, 1961, and abstracted in *Biochem. Pharmacol.* **8**, 72 (1961).

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periods ranging from a few weeks to a few months, the cancers returned and were then resistant to attack by the analogs.

The effects of these same analogs on transplanted cancers were more marked. Permanent cures were produced[4] in about half the cases, but this was true only when the cancers were within five passages of the original donors. With repeated transplantation they became completely resistant to the action of the analogs. In the "early passage" transplants, where the drugs were curative, a study of the relationship between dose and response indicated that all the cancers might be suppressed if a large enough dose could be used. The results with the spontaneous cancers likewise suggested that if a larger dose or a more active analog could be used it might be possible to inhibit all the cancers. Because a larger dose was not practicable, a search for more active congeners was made.

In this paper we wish to report the finding of some analogs of dimethyldiaminobenzene which were able to cure permanently some of the spontaneous mammary cancers of mice. These drugs are not able to cure all the cancers in a group of mice and hence they leave much to be desired, but they represent an advance over the ones previously described, in that they will bring about permanent cures of some spontaneous cancers. This advance came from finding compounds with higher potency as well as from improvements in the mode of administration. Because the problem of chemotherapy revolves around the cure of spontaneous cancers, not transplants mentioned above, all the testing was done in mice bearing spontaneous cancers, even despite the difficulties of obtaining large numbers of such animals.

The analogs that showed greatest potency in the present study were 1,2-dimethyl-4-(*p*-carboxyphenylazo)-5-hydroxybenzene or CPA, 1,2-dichloro-4-benzenesulfonamido-5-nitrobenzene or DCBN, and 1,2-dimethyl-4,5-*bis*(benzenesulfonamido) benzene or DMDB. The chemical structures of these compounds are shown in Fig. 1.

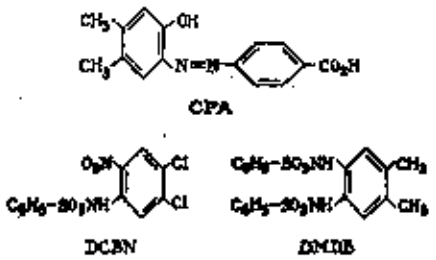

FIG. 1. Structures of the active agents.

As was described in the preceding papers of this series,[5, 7–8] the present compounds would not be expected to be reversed in their biological effects by vitamin B_{12}. They were designed with the express purpose in mind of avoiding such antagonism, because it was realized that if such antagonism did exist the vitamin in the food and tissues of the animals would prevent the therapeutic effect. Consequently, no effort was made to demonstrate reversal of the therapeutic effect on cancers; experiments with microorganisms had already demonstrated the failure of such reversal.[7, 8] The failure of vitamin B_{12} to reverse the therapeutic action is thus comparable to the failure of folic

acid to reverse the action of sulfanilamide in bacterial infections of animals. Because of this feature, which had intentionally been built into the oncolytic compounds, there was no direct demonstration that their effects on cancers were the result of interference with the biosynthesis of vitamin B_{12}. There was only the probability that this may have been so.

MATERIALS AND METHODS

Antimetabolites

CPA was synthesized according to the method of Woolley.[7] DCBN, DMBD, and their relatives were synthesized by methods to be described in a separate paper. Aminopterin was the product sold by Lederle Laboratories; 6-mercaptopurine was from the Burroughs-Wellcome Co. 5-Fluorouracil and 5-fluorodeoxycytidine were kindly supplied by Dr. R. Duschinsky of Hoffmann-La Roche, Inc.

Each analog to be administered orally was added in the desired amount to powdered stock ration (Purina Foxchow) together with enough $NaHCO_3$ or NaOH to yield the sodium salt of those compounds that were acids.* This mixture was then powdered in a ball mill and fed *ad libitum*. For parenteral administeration each compound was suspended by ball milling in Ringer's solution, or dissolved in enough NaOH to yield a neutral aqueous solution. The solutions were sterilized by heating and injected once daily intraperitoneally.

Animals

Two strains of mice bearing spontaneous mammary cancers were used (SPFS and C3H). The scarcity of *spontaneous* cancers limited the size of the experimental groups. The SPFS were Rockefeller-Institute-Swiss mice, all derived from three original litters which had been delivered by Cesarian section and maintained in isolation as a colony free of certain infectious, endemic diseases of mice (SPF means "specific pathogen-free"). The females of this colony were examined weekly, and whenever a small tumor was found in any mouse she was started on an experiment. The nature of the tumors was determined from time to time by excision of the neoplasms and histological examination of sections of them. The C3H mice were purchased from the Jackson Memorial Laboratories and were from their inbred colony. Individuals bearing spontaneous tumors less than 1 cm in greatest dimension were selected and used.

Methods

Female mice bearing spontaneous cancers were caged individually and fed either stock ration (Purina Foxchow) or stock ration to which the compounds had been added as described above. The size of each cancer was measured (length, width, and height) with calipers once each week. Body weight was also determined each week. Because large numbers of mice were never available at the same time, it was necessary to take them whenever the tumors were discovered. Consequently, the controls (untreated with the compounds) were chosen by alternately selecting individuals for control and experimental groups. In most experiments, all mice (test as well as

* As an example, 1 kg of ground Foxchow was mixed with 6 g of CPA. A solution of 500 mg of DCBN in 15 ml of 1 N NaOH and 5 ml of ethanol was spread over this mixture, and the whole was ball milled for 24 hr.

control) were fed stock ration for the first week and if any showed a decrease in size of tumor, they were discarded. The reason for this will be discussed in the section on Results. All mice were kept under examination until they died, or until they had been free of cancer for at least 5 months. Each experimental group contained six animals, and experiments were repeated at suitable intervals. Whenever a cancer disappeared, treatment with the analogs was stopped and only stock ration was fed.

This cessation of treatment seemed to be important for permanence of cure. Previous studies with less potent analogs[3, 4] had shown clearly that, if the therapeutic agents were continued after disappearance of the cancers, the neoplasms always returned. Because of this prior experience, the procedure with the new compounds was as described, even though no direct test of the phenomenon of reappearance was made with them.

Criteria of cure

A permanent cure of a cancer was judged to have taken place when the neoplasm decreased in size, disappeared, and remained undetectable for at least five months. Disappearance was judged by failure to detect it by palpation. It was not unusual to find that a tumor might decrease in size, and disappear for weeks or months, only to return and to grow until the animal died. Such behavior was termed *transient cure*. Sometimes a cancer might decrease in size, but after a few weeks would begin to grow again; this was called *transient regression*. Decreases greater than 0·2 cm in any dimension that continued for longer than 1 week were considered significant. Mere slowing of the rate of growth or failure to grow was not considered to be a regression.

When a mouse had more than one cancer, it sometimes happened that the individual tumors responded differently. One neoplasm might decrease or even disappear, while another might continue to grow. When such a response was seen it was counted as a transient regression, even though one of the tumors had completely disappeared. In cases of multiple cancers, all the neoplasms were required to disappear before the mouse was considered to be cured and counted as a permanent cure.

RESULTS

Response of controls

The cancers of every one of the C3H mice which was fed only stock ration continued to grow and eventually killed the animal. There were no cases of spontaneous regression. With the SPFS mice the situation was less clear. Some of these tumors showed regressions, and a few disappeared permanently. For this reason great attention was given to control groups.

To determine whether all the tumors were mammary cancers, histological examination of sections of the tumors from 23 SPFS mice was made.* This showed that all neoplasms larger than 1 × 1 × 0·5 cm (15 tumors) were typical, malignant mammary cancers.

With the smaller tumors (less than 1 × 1 × 0·5 cm), some proved to be abscesses or other nonmalignant growth. The number of noncancerous tumors among these small ones varied considerably from experiment to experiment. Thus, in one experiment, six of eleven small tumors were noncancerous on histological examination, but

* We are greatly indebted to Dr. John B. Nelson of this Institute for the histological examinations.

in another experiment involving ten mice with small tumors, all grew slowly to great size and killed their hosts.

The ideal way to avoid inclusion of noncancerous growths, apparently, would be to employ only large tumors, and also to take a biopsy specimen from each at the start of the experiment, in order that all doubtful cases be eliminated. The difficulty with this plan was (a) the larger the tumor the more difficult it was to attain cures with the analogs, and (b) taking biopsy specimens from a small tumor endangered its continued existence as a growing cancer.

To minimize possible errors, the control mice were chosen so that the sizes of the tumors in the experimental groups were comparable to those in the control groups. All mice were kept for 1 week on stock ration, and any one with a tumor that failed to grow during this trial period was discarded. In this way most of the growths that were merely abscesses were eliminated.

Even when noncancerous growths were excluded, the problem of spontaneous regression of SPFS tumors was not solved. Such spontaneous regressions were observed in some tumors which had been proved by histological examination to be malignant cancers. Thus, mouse 1528 was first observed with a tumor that measured 0·9 × 1·1 × 0·7 cm; this neoplasm grew steadily for 4 weeks and then began to decline. When the tumor had grown smaller for 6 weeks (0·6 × 0·6 × 0·6 cm), it was excised, sectioned, and examined histologically. It was a typical malignant growth. The tables of data contain several examples of similar spontaneous regressions and cures.

The variation in rate of growth of spontaneous cancers was much greater than that found in transplanted cancers. For this reason the effects of the drugs could not be judged merely from measurements of growth rate of the cancers. In the C3H animals, which were an inbred strain, the variation in growth rate of the cancers was less than in the SPFS strain, so that some useful information was gained from a study of growth rates in the C3H strain.

TABLE 1. EFFECTS OF ORALLY ADMINISTERED CPA AND DCBN ON SPONTANEOUS MAMMARY CANCERS OF SPFS MICE

CPA (g/kg ration)	DCBN (g/kg ration)	Mice	Trans. regres.	Trans. cures	Perm. cures
0	0	80	13	2	6
10	0·5	17	5	1	4
6	0·5	36	10	4	11
3	0·5	15	2	1	5
3	0·25	6	1	0	1
3	1·0	14	6	1	3
3	2·0	5	1	0	1
3	0	6	1	1	1
1·5	0·25	6	1	1	0
0	0·5	4	0	0	1
0	2·0	6	2	0	2

Effects of orally administered CPA and DCBN on cancers of SPFS mice

The effects of CPA and DCBN (either singly or together) are shown by the data in Table 1. The data suggest that in combination these compounds were able to cure permanently about one-third of the mice and to affect favorably most of them. The optimal dose seemed to be 6 g of CPA and 0·5 g of DCBN per kg of ration. Increasing

the amount of CPA to 10 g/kg did not give improved response; in fact, such an increase may have diminished the response somewhat, although the number of animals was not great enough to establish this last point. At the 10-g level the mice did not eat the ration readily and, during the first week or two, ate so little that they lost weight. With continued exposure to the ration, however they learned to eat it, and regained their lost weight. The diminished intake at the start might have been partially responsible for the relatively poor response at this dose level.

A further factor may have contributed to the failure of the 10-g dose to improve upon that obtained with 6 g. All mice which ate the rations containing CPA, up to and including the 6-g level, apparently reduced the azo group in the molecule completely. No red color was excreted in their urine and their tissues (aside from their fur) were not stained with the azo compound; however, those mice which ate the ration containing 10 g CPA/kg excreted red urine. This may have indicated that these animals had not degraded all of the CPA ingested and that some escaped into the urine. Whether or not CPA, *per se*, is an anticancer agent, or requires metabolic change to become active, is not known.

Whenever a mouse experienced a regression of its tumor, and the cancer disappeared, the administration of CPA and DCBN was stopped. This was done because, in earlier experiments with drugs of this class,[3, 4] it had been found that if administration was continued after the neoplasms had disappeared, the cancers invariably returned. It was thought that this may have been because a drug-resistant strain of the cancer had arisen in the continued presence of the drug in the animal, or that drug-destroying enzymes had arisen in the host. Experimental evidence for such points of view has been already presented.[3, 4] Nevertheless; the need for cessation of therapy when the cancers disappeared was not examined in the cases of CPA plus DCBN. To minimize risk of the development of such resistance, the discontinuance of the drugs was practised as outlined.

In several of the mice given CPA and DCBN the cancers disappeared, and administration of the drugs was stopped. After a few weeks or months the cancer again appeared. The compounds were not started again, but the growth of the new neoplasms was followed. In some cases they continued to grow and resulted in death of the hosts, but in others they receded again after 6 to 8 weeks and totally disappeared. This secondary disappearance was thus without direct aid of chemotherapy. It is possible that these animals were immune to the secondary cancer and had developed the immunity as a result of their previous temporary cure. No proof of such a possibility has been attempted. It is very difficult to provide adequate controls for experiments of this sort, because the mice which had not been treated with the drugs did not show the phenomenon.

Relative effectiveness of CPA and DCBN on large and small cancers of SPFS mice

Table 2 shows the relative effectiveness of CPA and DCBN fed together to SPFS mice bearing small tumors (less than 1 × 1 × 0·5 cm) and to those bearing larger tumors (greater than 1 × 1 × 0·5 cm). It was plain that, the larger the tumors when chemotherapy was started, the less chance there was for a cure. This same result was found with C3H cancers in which, although no cures were achieved, transient regressions of small cancers, but not of large ones, were found. Likewise, the same

phenomenon of the importance of starting with small cancers was seen when drugs such as DMDB were given to SPFS mice.

Effect of injected CPA and DCBN on spontaneous cancers of SPFS mice

CPA and DCBN cured some, but not all, mice when they were injected rather than fed. For example, three of six SPFS mice permanently lost their cancers when they were injected daily with 2·5 mg CPA and 0·5 mg DCBN. DCBN by itself cured three of seven mice at 1 mg/day, and one of ten mice at 0·5 mg/day, and CPA by itself (5 mg/day) cured none of the five mice permanently. The scarcity of mice with spontaneous cancers limited the number of animals tested, but the results showed clearly that injection (rather than feeding) of the compounds would not cure all of the cancers.

TABLE 2. RELATIVE EFFECTIVENESS OF CPA AND DCBN ON LARGE AND SMALL CANCERS OF SPFS MICE

Large cancers were greater than 1 cm and small cancers less than 1 cm at the start of the experiment.

CPA (g/kg ration)	DCBN (g/kg ration)	Mice with small c.	Mice with large c.	Perm. cures
0	0	37		6
0	0		43	0
10	0·5	12		4
10	0·5		5	0
6	0·5	19		7
6	0·5		17	4
3	2		5	1
3	1	5		2
3	1		9	1
3	0·5	12		5
3	0·5	3		0
3	0·25	3		1
3	0·25		3	0
3	0	4		2
3	0		2	0
1·5	0·25	6		0
0	2	3		2
0	2	3		0
0	0·5		4	1

TABLE 3. EFFECTS OF ORALLY ADMINISTERED CPA AND DCBN ON SPONTANEOUS MAMMARY CANCERS OF C3H MICE

CPA (g/kg ration)	DCBN (g/kg ration)	Mice	Trans. regres.	Trans. cures	Perm. cures
0	0	54	0	0	0
3	0·5	17	6	1	0
6	0·5	17	10	0	0

Effect of orally administered CPA and DCBN on C3H spontaneous cancers

The data of Table 3 summarize the effects of CPA and DCBN when fed to C3H mice bearing small, spontaneous mammary cancers. No permanent cures were observed, but in one mouse a transient cure occurred. This one case seemed significant because in this strain of mouse the cancers never failed to grow steadily, and spontaneous cures were never observed. Furthermore, the compounds brought about

transient regressions of the cancers in many of the mice during the first weeks of the experiment. This indicated that these compounds are active but not active enough to succeed in this strain. Fig. 2 shows the average growth rate of the cancers in six treated mice, as compared with that in six untreated controls. The transient regressions (the dip in the curve of Fig. 2) were not just the result of feeding some noxious compound that may have made the mice sick and thus unable to support the normal growth of their cancers. A very large experience with many kinds of chemical substances fed to C3H mice during the past 14 years has shown us that the tumors continue to grow whether the host is well or ill. Furthermore, the mice fed CPA and DCBN were not ill, insofar as could be detected; they grew and reproduced normally. The results shown in Fig. 2 were confirmed in two additional experiments.

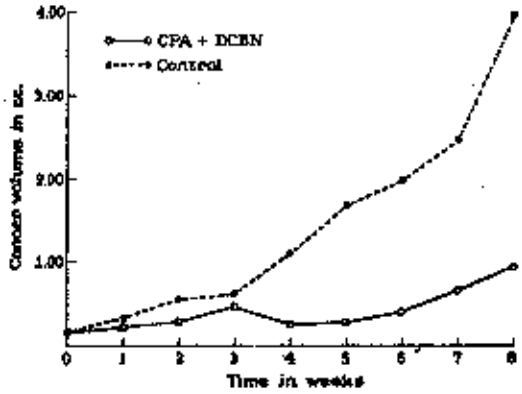

Fig. 2. Average growth rates of spontaneous mammary cancers of C3H mice fed CPA + DCBN or control rations. Average was obtained from 6 mice in each group. The drug ration contained CPA at 6 g/kg and DCBN at 0·5 g/kg.

Effect of DMBD on SPFS spontaneous mammary cancers

Table 4 shows the results of feeding or injecting DMDB into SPFS mice bearing spontaneous mammary cancers. There was some curative action, but it was less than with CPA plus DCBN. The addition of CPA (3 g/kg ration) to DMDB (5 g/kg) did not increase the percentage of cures.

Effect of DMDB on spontaneous C3H cancers

C3H mice bearing small spontaneous mammary cancers were treated with DMDB in the manner described for the SPFS mice. Both oral and intraperitoneal routes were studied. The results summarized in Table 4 show that this compound caused transient regressions but no permanent cures. The indication was that it is probably a more potent inhibitor of tumor growth in C3H than in SPFS cancers but is not active enough to cure significant numbers of either strain.

Lack of toxicity to the host animals

When given by the oral route CPA, DCBN, or DMDB did not exhibit significant toxicity, either for normal mice or for the cancerous ones. Except for those getting 10 g of CPA plus 0·5 g of DCBN per kg of ration, all animals ate the rations readily and suffered no loss of body weight or other signs of illness from the compounds.

Even those animals receiving 10 g of CPA plus 0·5 g of DCBN learned eventually to eat the ration, and then showed no signs of toxic effects; their lack of toxicity was indicated further by their ability to bear normal litters. The achievement of selective toxicity, such that the drugs would harm the cancers without at the same time harming the host animals, has been the object of the present approach to the chemotherapy of this disease, as outlined in the earlier papers.

TABLE 4. EFFECTS OF FED OR INJECTED DMDB ON THE SPONTANEOUS MAMMARY CANCERS OF SPFS AND C3H MICE

DMDB p.o. (g/kg ration)	DMDB i.p. (mg/mouse/day)	Mice	Trans. regres.	Trans. cures	Perm. cures
		SPFS mice			
0	0	80	13	2	6
5	0	7	2	0	1
0	3	16	3	0	2
		C3H mice			
0	0	54	0	0	0
5	0	19	6	0	0
0	2·5	31	16	1	0

TABLE 5. EFFECTS OF RELATED DRUGS ON SPONTANEOUS MAMMARY CANCERS OF MICE

Drug*	p.o. (g/kg ration)	i.p. (g/mouse/day)	SPFS mice	C3H mice	Trans. regres.	Trans. cures	Perm. cures
DCDB	0	2·5	0	11	5	0	0
DCDB + CPA	2 + 3	0	0	12	7	4	0
DCDB + CPA	2 + 3	0	6	0	1	0	1
DCBA	0	1	9	0	3	0	2
DCBA + CPA	1 + 3	0	6	0	1	2	0
DCBS	0	4	0	8	2	0	0
DCBS + CPA	2 + 6	4	0	6	3	0	0
DCBS + CPA	2 + 6	4	7	0	2	0	2
DCBT	0	5	12	0	1	0	0
DCBT	0	5	0	12	1	0	0
DMBA	0	2·5	0	6	1	0	0
DMBA	0	2·5	6	0	3	0	0
DMBS	0	5	7	0	5	2	0

* DCDB was 1,2-dichloro-4,5-*bis*(benzenesulfonamido)benzene; DCBA was 1,2-dichloro-4-benzenesulfonamido-5-aminobenzene; DCBS was 1,2-dichloro-4-benzenesulfonamido-5-succinamidobenzene; DCBT was 1,2-dichloro-4-benzenesulfonamido-5-phthalimidobenzene; DMBA was 1,2-dimethyl-4-benzenesulfonamido-5-aminobenzene; DMBS was 1,2-dimethyl-4-benzenesulfonamido-5-succinamidobenzene.

Although the drugs were harmless when fed, they did have some toxicity when injected in large amounts. Levels double those reported in the tables occasionally caused death of the animals; however, the amounts used in the therapeutic experiments caused no loss of body weight or other toxic manifestations detectable by gross examination.

Effects of congeners of the active drugs on spontaneous cancers

Several congeners of DCBN and DMDB were tested in the ways just described. The results are summarized in Table 5. Although the numbers of animals employed with

each compound were too small to make possible an accurate assessment of the potency relative to the parent substances, the results showed clearly that none was markedly more active than the parent compounds. They did not cure all the mice. In fact, the suggestion was that most of them were less active than the parent compounds, or not active at all. The scarcity of mice with spontaneous mammary cancers made any further trials with these less potent compounds unjustifiable.

Failure of known oncolytic agents to cure spontaneous mammary cancers

Several chemical substances are known which have shown the ability to reduce the growth rate, or actually to cure, certain transplanted cancers of mice. Some of these have been tested to determine whether they would cure permanently the spontaneous cancers of SPFS and C3H mice. In every instance the compounds were administered daily by the intraperitoneal route, and the dose used was of maximal tolerance. Aminopterin (1 μg/mouse/day), 5-fluorouracil (0·3 mg/mouse/day), 5-fluorodeoxycytidine (1·0 or 1·3 mg/mouse/day), and 5-fluorodeoxyuridine (1 mg/mouse/day) each failed to cure any mice. The experience with spontaneous cancers of mice thus paralleled the results which others have found in spontaneous cancers of human beings, and did not coincide with those found with transplanted cancers of mice.

DISCUSSION

The data presented suggest that a mixture of CPA and DCBN brought about permanent cures of spontaneous mammary cancers of some mice of the SPFS strain. This mixture also caused some regressions of the spontaneous mammary cancers of C3H mice but did not permanently eradicate them. DMDB was less potent but did produce some curative effects. In the case of the SPFS cancers, the larger the tumor when treatment was begun, the more difficult it was to bring about a cure.

The fact that some of the untreated SPFS tumors exhibited spontaneous cure was disturbing and raised the question as to whether there really was any significant effect of the compounds at all. The fact that the compounds never cured all the cancers argued in favor of the idea that they really were not curative. However, opposed to such a point of view are the following considerations: (a) In each experiment with CPA plus DCBN, the number of cures was always greater in the treated than in the control groups. (b) Inactive compounds were readily distinguishable from the active ones; this would not have been the case if all compounds, including CPA plus DCBN, had been inactive. (c) With C3H cancers, in which the problem of spontaneous regressions never arose, the inhibitory action of CPA plus DCBN was still detectable; permanent cures of C3H cancers were not produced, but transient regressions and a transient cure were.

The ability of CPA plus DCBN to cause permanent cure of the spontaneous mammary cancers of SPFS mice represents the first case of the cure of such cancers by any chemical agent. Many other compounds, which have proved to be inhibitory or even curative of certain transplanted tumours, failed to affect these spontaneous ones, just as they have failed in spontaneous cancers of human beings. Nevertheless, the activity of CPA plus DCBN was not great enough to bring about cure of all cancers in a group of mice. The fact that as the dose was raised a plateau of response was reached, such that further increases in dose did not bring about any greater percentage of cures, may be interpreted in one of two ways. Either about half the mice bore cancers of a

different kind which were not susceptible at all to the compounds, or some property of these compounds, such as their insolubility in water at neutral pH, restricted their biological activity. Perhaps it will be possible to develop more potent congeners of these drugs that will cure all the mice with a reasonable dose.

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Change of the System for the Adsorption of Vitamin B_{12} to Intestinal Mucosa Homogenate in Growing Rats

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YAMADA, S., HORIO, S. and KIMURA, S. *Change of the System for the Adsorption of Vitamin B_{12} to Intestinal Mucosa Homogenate in Growing Rats.* Tohoku J. exp. Med., 1972, 106 (4), 363-371 —— Adsorption of vitamin B_{12}, with and without gastric mucosa extract (GME), to intestinal mucosa homogenates from rats was studied at various stages of growth. The development of the activities of producing gastric HCl and pepsinogen was also investigated. The intestine from newborn animals showed extremely high adsorption of vitamin B_{12} in the absence of GME. The GME-independent vitamin B_{12} adsorption fell rapidly with time, approaching adult level at about 20 days of age. Stimulation of vitamin B_{12} adsorption by adult GME and the gastric HCl secretion reached adult levels at about 20 days. On the other hand, intrinsic factor formation and pepsinogen formation did not reach adult levels until about 30 days. Histologic investigations of the gastric gland and ileal villus agreed with the above-mentioned experimental results. The intrinsic factor-dependent vitamin B_{12} absorption became a predominant system at about 30 days after birth. ——— vitamin B_{12} absorption; intrinsic factor

In our preceding papers (Yamada *et al.* 1970, 1971), we reported the results obtained by comparative studies on the adsorption of vitamin B_{12} to intestinal mucosa homogenates of adult and unweaned rats (48 hours after birth). The data obtained indicated that 1) stomach of the unweaned rats secreted neither intrinsic factor (IF) nor IF-like materials, 2) the amount of vitamin B_{12} adsorbed to the intestinal mucosa homogenates of the unweaned rats in the absence of IF was very high compared with that to the intestinal mucosa homogenates of the adult rats, and 3) adsorption of vitamin B_{12} to the intestinal mucosa homogenates of the unweaned rats was not significantly affected by the omission of bivalent cations from the incubation medium. These results suggested that IF-independent vitamin B_{12} absorption is predominant in the unweaned rats. In the present paper we report the results of the investigation which aimed to elucidate when the IF-independent vitamin B_{12} absorption system would be converted to the IF-dependent absorption system in the course of neonatal life to maturation. The development of intestinal receptor for IF-vitamin B_{12} complex was also examined in relation to the development of IF secretion. Studies were also made on the relations between the time

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of onset of IF secretion and those of some gastric secretions other than IF, *i.e.* gastric HCl and pepsinogen.

Methods and Materials

1) *Experimental materials*

a) *Animals.* Wistar strain male albino rats fed on commercial diet (NMF; product of Oriental Yeast Co. Ltd.) were used throughout the experiments. Young rats before 37 days of age were permitted to nurse until sacrification. Rats aged five months or more were used as adult in this study. We considered it necessary to empty the stomach and small intestine in order to eliminate the effects of vitamin B_{12} in the diet or rat's milk and to make the gastrointestinal tract of individual animals uniform. So, for the measurement of IF activity and determination of pepsinogen in gastric mucosa and for the measurement of the amount of vitamin B_{12} adsorbed to intestinal preparations, all rats were fasted for from 6 to 24 hours. After sacrification by decapitation, stomach and small intestine were resected immediately for the preparations as described by Castro and Glass (1963, 1964). All preparations were stored in a freezer at −20°C until use. Since it was difficult to obtain intestinal preparations from mucosal scrapping alone with rats younger than 15 days of age, the small intestine was homogenized with its serosa in these rats. Whole glandular portion of the stomach was used throughout all the periods.

b) *Radioactive vitamin B_{12}.* ^{57}Co-cyanocobalamin (Radio Chemical Centre, Amersham, England) with specific radio-activity of 4.4 μCi/μg was used.

2) *Methods*

The amounts of vitamin B_{12} adsorbed to intestinal mucosa preparations and IF activities of gastric mucosa preparations were measured according to the method by Castro and Glass (1963, 1964). IF activities were expressed as percentage of that in the adult rats. The amount of the preparations used were as follows; intestinal preparations, 10 mg of lyophilyzed preparations; gastric preparations, 1 mg wet weight of gastric glandular portion. Pepsinogen in the gastric mucosa was determined by Hagihara's modification (1956) of Anson and Mirsky method (1932, 1938) after being converted to pepsin by adjusting the pH of the gastric mucosa extract to 1.8 with HCl-KCl buffer solution. Crystalline pepsin (Pepsin, 3X crystallina, NBC, Co. Ltd.) was used as reference. pH values in the stomach was measured with pH test paper. For histologic investigations, the fundus portion of the stomach and lower ileum were fixed by Bouin's fluid, mounted in paraffin, sliced by microtome and stained by hematoxylin-eosin.

Results

Development of intestinal receptor for IF-vitamin B_{12} complex

Fig. 1 shows the changes of the amount of vitamin B_{12} adsorbed to intestinal preparations without IF along with the days after birth. The value decreased sharply until 20 days, approaching the adult level. In Fig. 2 are shown the effects of addition of the gastric mucosa extract of the adult rat on the adsorption of vitamin B_{12} to the intestinal preparations of the rats of various ages. Adult level was reached in 20-day-old rats. The results mentioned above suggest that functions concerning the absorption of vitamin B_{12} in the intestine in 20-day-old rats are almost the same as those in the adult rats.

Beginning of formation of IF in the stomach

Fig. 3 shows the effects of addition of the gastric mucosa extract from the rats

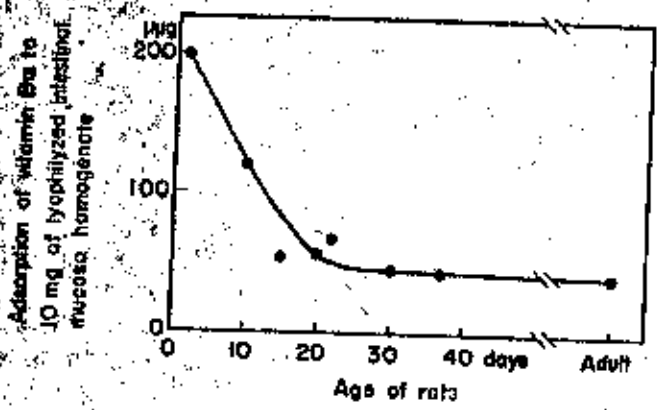


Fig. 1. Adsorption of vitamin B_{12} to the intestinal mucosa preparations obtained from rats of various ages in the absence of IF.

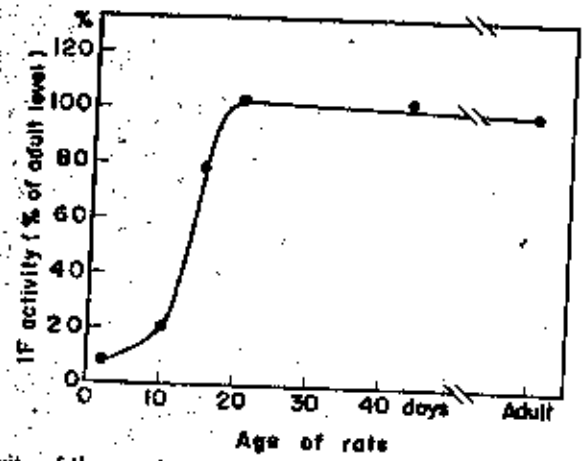


Fig. 2. IF activity of the gastric preparations of adult rats on the intestinal preparations obtained from rats of various ages.

of various ages on the vitamin B_{12} adsorption to the intestinal preparation of the adult rats. The activity increased rapidly after about 20 days of age and reached adult level at about 30 days after birth.

Vitamin B_{12} adsorption at various ages

Experimental results cited above indicate that the intestinal receptor for IF-vitamin B_{12} complex is completed at about 20 days of age and the secretory activity of IF reaches the adult level at about 30 days. Then, it was examined how effective the gastric mucosa extracts of rats of various ages are on the vitamin B_{12} adsorption to the intestinal preparations of the rats of the respective same ages. As shown in Fig. 4, the activity began to rise at about 20 days of age, approaching the adult level at about 34 days.

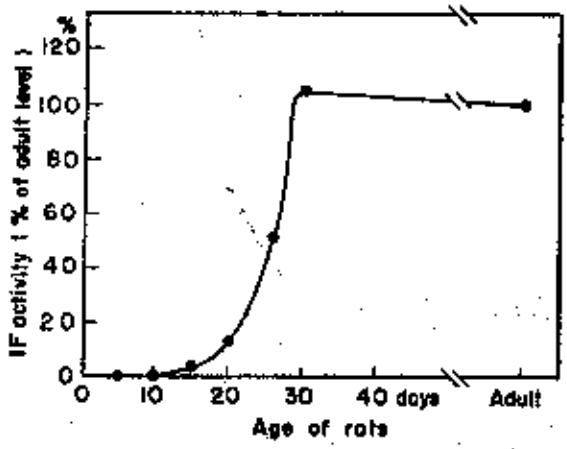


Fig. 3. IF activity of the gastric preparations obtained from rats of various ages on the intestinal preparations from the adult rats.

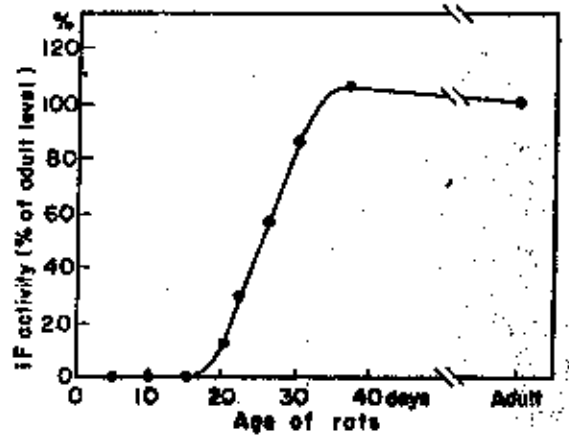


Fig. 4. IF activity of the gastric preparations obtained from the rats of various ages on the intestinal preparations from rats of respective same ages.

pH value in the stomach and pepsinogen formation

pH value in the stomach decreased gradually with growth and reached nearly the adult level at 20 days after birth (Fig. 5). Pepsinogen, resembling the development of adult level IF shown in Fig. 3, began to increase rapidly at 20 days and reached the at 30 days (Fig. 6).

Histologic findings on gastric mucosa and lower ileum

Fig. 7 shows histologic findings on gastric mucosa of rats at 2, 20, 30 days and adult and on the lower ileum of rats at 2, 15, 20 and 30 days and adult.

1) *Stomach* In the rats at 2 days of age (Fig. 7-A) only mucous neck cells were seen, but neither parietal cells nor chief cells could be seen. At 20 days, parietal cells have been developed well, whereas chief cells have not been

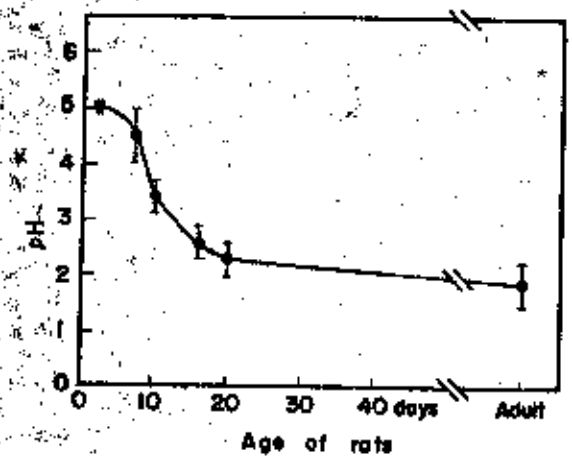


Fig. 6. pH value in the stomach of rats of various ages.

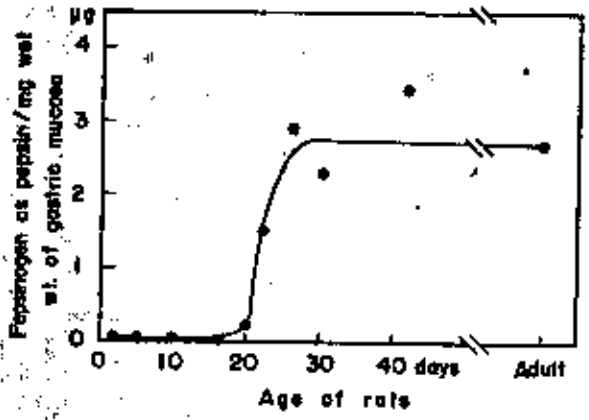


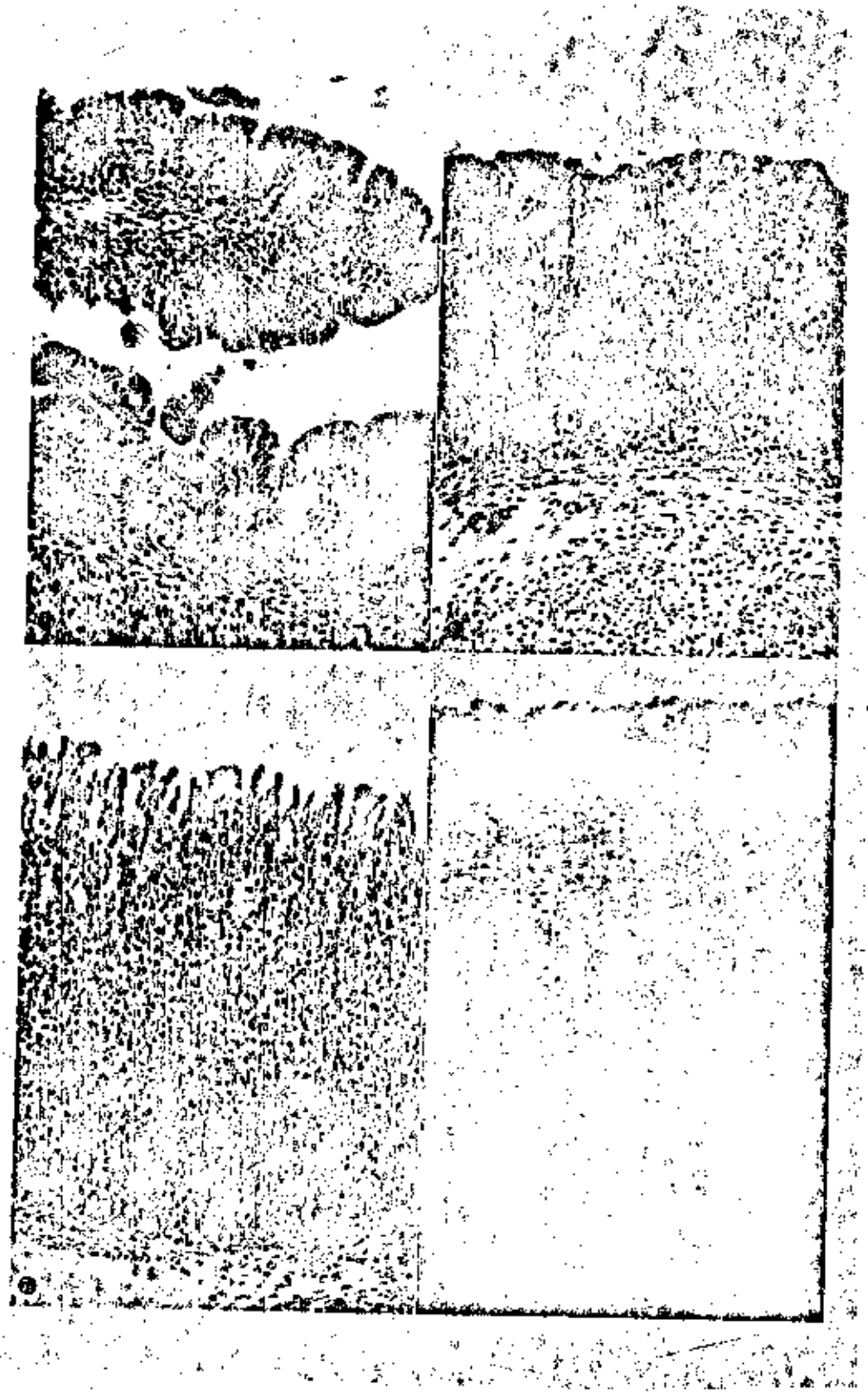
Fig. 8. Development of pepsinogen formation in rats of various ages.

developed adequately (Fig. 7-B). At 30 days of age (Fig. 7-C), both parietal and chief cells have developed well and the appearances were the same as those in the adult rats (Fig. 7-D).

2) *Lower ileum.* In 2-day-old rats, a great number of glycoprotein granules were seen uniformly, suggesting that the absorptive functions were far from perfect (Fig. 7-E). At 15 days (Fig. 7-F) polysaccharide granules were localized at the top of villi. This indicates that their functions were still inadequate. In 20-day-old rats (Fig. 7-G), villi were still shorter than those in 30-day-old rats (Fig. 7-H) or adult rats (Fig. 7-I), but the development of epithelial columnar cells were adequate. These findings agree well with experimental results described earlier.

Discussion

The results obtained in the present studies suggest that IF-independent vitamin



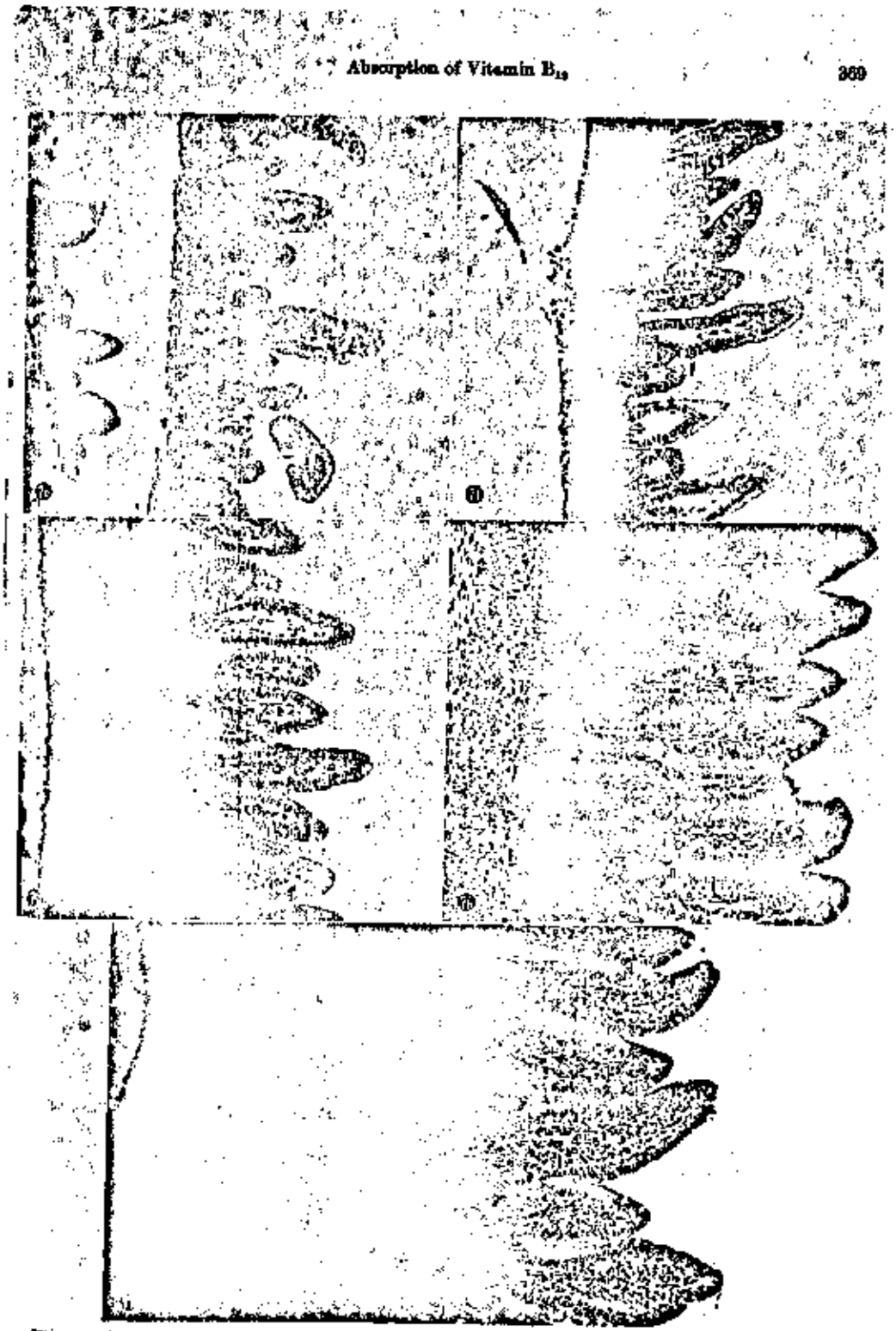


Fig. 7. Histologic findings on gastric gland and ileum. Hematoxylin-eosin staining, 20 × 5. a: gastric gland, 2 days old. b: gastric gland, 20 days old. c: gastric gland, 30 days old. d: gastric gland, adult. e: ileum, 2 days old. f: ileum, 15 days old. g: ileum, 20 days old. h: ileum, 30 days old. i: ileum, adult.

B_{12} absorption, which represents the predominant system in the rat until about 20 days of age, changes gradually to the IF-dependent system in the course of life between 20 and 30 days. The results shown in Figs. 1, 3 and 6 support the findings reported by Boass and Wilson (1963). They reported that IF-independent vitamin B_{12} uptake was reduced to the adult level at 14 days of age. On the other hand, our experimental results shown in Figs. 1 and 2 indicate that functions of small intestine concerning the vitamin B_{12} absorption did not attain adult level until about 20 days. Moreover, histologic finding suggest that the ileum of the 15-day-old rats did not have the same functions as those of the adult rats. Halliday (1954) reported that in young rats intestinal absorption of antiserum decreased rapidly at about 20 days after birth. Weaning period of rat is known to be about 20 days. Putting all accounts together, it may be rational to consider that the mechanism of vitamin B_{12} absorption changes at about 20 days of age rather than 14 days.

Boass and Wilson (1963) stated that IF-independent vitamin B_{12} uptake reached the adult level at 14 days, and at that time gastric IF activity was 30% of the adult level; it took 3 weeks until the gastric IF activity reached the adult level. In our experiments, however, the IF-independent vitamin B_{12} adsorption reached the adult level at about 20 days and the gastric IF activity at this age is a little less than 14% of the adult level. Gastric IF activity did not attain adult level until about 30 days of age. The experimental results of Boass and Wilson (1963) as well as ours showed that there was a time-discrepancy between the maturation of absorptive function and the secretory function, and that in a certain period during the conversion from the IF-indpendent vitamin B_{12} absorption to the IF-dependent vitamin B_{12} absorption, there appeared a pocket, namely the period when both absorptive capacities were low. But *in vitro* experiments as reported by Boass and Wilson (1963) and by us would reflect only a part of total absorptive capacity in both IF-independent and IF-dependent systems. Therefore, the results of *in vivo* experiements are of interest. Williams and Spray (1968) reported the studies *in vivo* in rats. Their results indicate that the percentage of test dose of cyanocobalamin (0.05 mμg/g body weight) absorbed, when administered orally, decreased gradually with age after 8 days and there was no pocket.

Generally, it has been known that vitamin B_{12} in blood of adult animals is bound to a protein-like material and that receptors for this complex are present in many organs and tissues. About the origin of the protein-like material binding vitamin B_{12} in blood, there has been two theories. One is that this material is derived from gastric IF, the other is that gastric IF cannot be absorbed across the intestinal mucosa and this material is synthesized *de novo* in the intestine to bind vitamin B_{12}. Assuming that the former is true, in the newborn rats, in which vitamin B_{12} is absorbed well without the aid of IF, vitamin B_{12} in blood may be in free form. In such a case, it is an interesting problem whether or not the receptors for the complex of vitamin B_{12} and protein-like material are present in organs and tissues. From the fact that pH change in the stomach precedes the beginning of

formation of pepsinogen and IF, it is clear that the development of gastric parietal cells is faster than that of chief cells. This was also confirmed by hitologic findings. The principal role of the gastric HCl is to convert the proenzyme pepsinogen to pepsin and to adjust the pH value in the stomach to an optimal pH for pepsin. It is obscure at present whether the fact that HCl formation precedes pepsinogen formation indicates only the difference in differentiation of secreting cells or some physiological significance. The development of pepsinogen formation resembles that of IF, another secretion from chief cells. There may be a common step in the synthesizing process of these two materials.

Acknowledgment

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CHEMICAL AND BIOCHEMICAL STUDIES ON VITAMIN B_{12} AND ITS RELATED COMPOUNDS

XVI. FACTORS AFFECTING THE ACTIVITY OF A DIOLDEHYDRASE REQUIRING COBAMIDE COENZYME. ENHANCEMENT BY ANAEROBIC LIGHT-IRRADIATION AND COMPETITIVE INHIBITION BY ADENOSINE

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The studies on the photochemical decomposition of cobamide coenzyme have made great contributions not only to the elucidation of its structure but also to the study on its reaction mechanism. The formation of methionine from homocysteine and methylcobalamin, an artificial analogue of the coenzyme, by illumination (Johnson *et al.* (1)) led to the finding of the effectiveness of the compound in an enzymic synthesis of methionine (Guest *et al.* (2), Weissbach *et al.* (3)), in which protein-bound B_{12s} or B_{12r} is supposed to act as a methyl carrier (Kerwar *et al.* (4), Brot *et al.* (5)).

In the enzymic conversion of 1,2-propanediol to propionaldehyde catalyzed by a dioldehydrase requiring the coenzyme, a direct intramolecular 1,2-hydrogen shift has been demonstrated by Brownstein and Abeles (6) and Frey *et al.* (7). There are many reactive positions supposed to be active for hydrogen transport in the coenzyme molecule. Among them the most probable one is a reduced state cobalt atom of the protein-bound B_{12r} or B_{12s} resulting from the cleavage of the cobalt-carbon bond linking a 5'-deoxyadenosyl moiety, even though some hydroxyalkyl cobalamins presumed to be active intermediates, for example, β,γ-dihydroxypropyl-cobalamin, were inactive in the dioldehydrase system (8). The present paper describes experiments concerning the effects of illumination as well as adenosine on the dioldehydrase activity under various conditions to obtain further information on the active sites.

MATERIALS AND METHODS

An authentic sample of DBCC[2] crystals was generously given by Prof. H.A. Barker and Dr. D. Perlman. 2',5'-Dideoxyadenosylcobalamin was kindly supplied from Dr H. P. C. Hogenkamp. Methylcobalamin was synthesized according to the method of Bernhauer *et al.* (9). Adenosine, inosine, adenine, AMP and DL-propanediol were

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[2] The following abbreviation is used: DBCC, 5,6-dimethylbenzimidazolyl cobamide coenzyme.

obtained from commercial sources. Apoenzyme used in this study was prepared from the cells of *Aerobacter aerogenes* (ATCC 2784) according to the procedure of Lee and Abeles (10), except that propanediol was not added, which was used as a stabilizing agent by the original authors. The properties of the apoenzyme were essentially identical with those of an authentic sample supplied generously by Prof. R.H. Abeles. It showed similarly a single migration zone on paper electrophoresis as reported by the original authors (10) and gave a specific activity, about 40 units per mg protein. The enzymic transformation of propanediol to propionaldehyde was studied according to Abeles and Lee (11).

The influences of irradiation on the propionaldehyde formation were examined under the following conditions: (*a*) apoenzyme and DBCC or its analogue were preincubated for 10 minutes at 30° in the dark, then propanediol was added and incubation was done for 10 minutes at 30° in the dark (control); (*b*) apoenzyme and the coenzyme or its analogue were preincubated for 7 minutes at 30° in the dark, then irradiated for 3 minutes at 30° prior to the incubation with the substrate; (*c*) the apoenzyme-coenzyme mixture was irradiated for 5 minutes at 0° prior to the incubation with the substrate; (*d*) the mixture of apoenzyme, DBCC and propanediol was incubated for 10 minutes at 30° in the dark, then further incubated for 10 minutes with irradiatien under the same conditions. These treatments were done under anaerobic conditions and in the presence of air, respectively. Anaerobic experiments were carried out using a Thunberg tube in H_2 atmosphere. Exposure to light was performed using a 300-watt tungsten lamp at a distance of 15 cm. The change of the absorption spectra was studied in a Thunberg tube-connected cuvette using Bausch-Lomb Spectronic 20 Colorimeter.

RESULTS

1. Effect of Light Irradiation after Holoenzyme Formation

When apoenzyme and DBCC were preincubated for 7 minutes at 30° in the dark, then irradiated for 3 minutes prior to the incubation with the substrate (condition *b*), the enzymic activity increased about twice under anaerobic condition. On the other hand, it decreased to less than a half under aerobic condition. Without the illumination the existence of air did not affect the reaction rate (Table 1, Expt. 1).

It has been known that DBCC readily decomposes by illumination to hydroxocobalamin an adenosine-5'-aldehyde-(Hogenkamp, *et al.* (12)) or adenosine-5'-carboxylic acid (Johnson and Shaw (13)) under aerobic conditions and to B_{12r} and 5'-deoxyadenosyl radical (Hogenkamp, *et al.* (14)) followed by the formation of 8,5'-cycloadenosine under anaerobic conditions (15, 16), respectively. These facts suggest that the stimulating effect of irradiation is due to an anaerobic cleavage of the cobalt-carbon bond forming a protein-bound B_{12r} and 5'-deoxyadenosyl radical, even if the protein-bound B_{12r} may be further reduced to the state of B_{12s} before reaction with the substrate.

The accelerating effect of illumination under anaerobic conditions was more evident when 2',5'-dideoxyadenosyl cobalamin was used (Table 1, Expt. 2). The coenzyme activity of the compound was significantly lower than that of DBCC

TABLE 1

Effect of Illumination on Dioldehydrase Activity Incubated with DBCC and 2',5'-Dideoxyadenosyl Cobalamin prior to Addition of Substrate

Expt. No.	Coenzyme	Irradiation	Condition	Relative activity
				per cent
1	DBCC	−[a]	Aerobic	100
		−	Anaerobic	113
		+[b]	Aerobic	42
		+	Anaerobic	190
2	2',5'-Dideoxy-adenosyl cobalamin	−[a]	Aerobic	40
		−	Anaerobic	40
		+[b]	Aerobic	12
		+	Anaerobic	196

The final reaction mixture contained in a total volume of 1.0 ml: apoenzyme, 0.09 units (2.1 mg); coenzyme, 2.0 mμmoles; DL-propanediol, 10 μmoles; potassium phosphate buffer (pH 8.0) 20 μmoles.

[a] Apoenzyme and coenzyme or the analogues were preincubated for 10 min at 30° in the dark then substrate was added and incubated for 10 min at 30° in the dark (condition *a* in the text), under aerobic or anaerobic conditions, respectively.

[b] Apoenzyme and coenzyme and the analogues were preincubated for 7 min at 30° in the dark, then illuminated for 3 min at 30°. After the substrate was added, the mixture was incubated in the dark (condition *b* in the text).

under ordinary assay conditions. Hogenkamp *et al.* (17) attributed the low activity to the resistance of its cobalt-carbon bond to the cleavage in the enzymic reaction caused by the change of the polarization of the bond. This compound, however, exhibited almost the same activity as DBCC when irradiated under anaerobic conditions prior to incubation with the substrate. The result would be explained by the acceleration of the cleavage of its cobalt-carbon bond by light irradiation, followed by the formation of B_{12r} and 2',5'-dideoxyadenosyl radical, at least as an intermediary state.

2. Effect of Light Irradiation before Holoenzyme Formation

The acceleration caused by the anaerobic photolysis was observed only with holoenzyme irradiated prior to the substrate addition. When light was irradiated at 0° (condition *c*) under the anaerobic condition where most of the apoenzyme and DBCC were considered to be present in a separate state, then incubated for 10 minutes at 30°, whereby the coenzyme activity decreased to the same extent as that of the aerobic photolysis of the holoenzyme mentioned above (Table 2, Expt. 1). In this case a significant part of free DBCC appears to be decomposed to hydroxocobalamin.

3. Effect of Light Irradiation after the Start of Enzyme Reaction

When illumination was carried out at 10 minutes after the addition of the substrate to the holoenzyme, where the enzymic reaction began to proceed steadily (condition *d*), light did not affect the reaction rate (Table 2, Expt. 2).

4. Effect of Light Irradiation on the Incubation Mixture of Methylcobalamin with Apoprotein

The observations described above suggest that the accelerating effect of light

TABLE 2

Effect of Illumination before the Holoenzyme Formation or after the Incubation with Propanediol

Expt. No.	Irradiation	Condition	Relative activity
			per cent
1	−[a]	Aerobic	100
	−	Anaerobic	105
	+[b]	Aerobic	40
	+	Anaerobic	42
2	−[c]	Aerobic	100
	−	Anaerobic	101
	+[d]	Aerobic	97
	+	Anaerobic	97

The reaction mixture contained in a total volume of 1.0 ml: apoenzyme, 0.10 units (2.4 mg); DBCC, 3.5 mμmoles; DL-propanediol, 1.0 μmoles; potassium phosphate buffer (pH 8.0), 20 μmoles.

[a] The reaction mixture was incubated for 10 min at 30° in the dark.

[b] The mixture of the apoenzyme and DBCC was irradiated for 5 min at 0° prior to the substrate addition. Then incubated with the substrate for 10 min at 30° in the dark (condition *c* in the text).

[c] The mixture of the holoenzyme and the substrate was incubated for 20 min at 30° in the dark.

[d] The mixture of the holoenzyme and substrate was incubated for 10 min at 30° in the dark, then 10 min under illumination (condition *d*).

TABLE 3

Effect of Illumination on Dioldehydrase Preincubated with Methylcobalamin

Irradiation	Condition	Relative activity
		per cent
−	Aerobic	6
−	Anaerobic	10
+	Aerobic	4
+	Anaerobic	14

The final reaction mixture contained in a total volume of 1.0 ml: apoenzyme, 0.09 units (2.1 mg); methylcobalamin, 2.0 mμmoles; DL-propanediol, 10 μmoles; potassium phosphate buffer (pH 8.0) 20 μmoles. The conditions were similar to those in Table 1.

is due to the formation of protein-bound B_{12r} or B_{12s} which takes up a hydrogen or a proton from the substrate.

However, the effect of the light irradiation was not observed in the case of methylcobalamin (Table 3). Methylcobalamin is known to act a competitive inhibitor in dioldehydrase system (18, 19) and to be converted to B_{12r} by anaerobic irradiation (20). From these facts it is considered that methylcobalamin can combine with the apoprotein, yielding protein-bound B_{12r} or a further reduced state, B_{12s}, by illumination. Nevertheless, light irradiation did not exert the stimulating effect in this case.

5. Effect of Adenosine or the Analogues on Dioldehydrase Activity

The ineffectiveness of illumination on the incubation mixture of methylcobalamin with apoprotein may suggest that the enzymic reaction cannot be mediated only by protein-bound B_{12r} or B_{12s} alone but that protein-bound 5'-deoxyadenosyl

TABLE 4
Effect of Adenosine or its Related Compounds on Dioldehydrase Activity

Addition	Concentration	Relative activity
	μmoles	*per cent*
Adenosine	0	100
	10	83
	50	75
	100	61
	150	50
	200	41
AMP	150	80
2'-Deoxyadenosine	150	87
Inosine	150	91
Adenine	150	91

The incubation mixture was: apoprotein, 0.09 units (2.0 mg); DBCC 1.0 mμmole; DL-propanediol 5 μmoles; potassium phosphate buffer (pH 8.0) 20 μmoles and additions as indicated above. Total volume 1.0 ml. Incubated for 10 min at 30° in the dark.

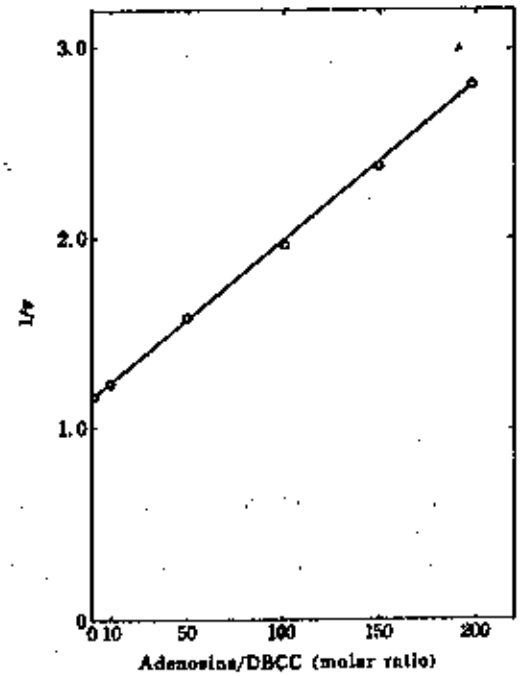

FIG. 1 *Competitive Inhibitory Effect of Adenosine on Dioldehydrase Requiring DBCC in the Conversion of Propanediol to Propionaldehyde.*
The experimental conditions are the same as those described in Table. 4.

radical or 2',5'-dideoxyadenosyl radical is also necessary for the reaction.

The participation of 5'-deoxyadenosyl moiety in the coenzyme activity could be examined by the effects caused by the addition of the compound having the structure analogous to that of the radical. Adenosine exhibited obviously a competitive inhibitory action with 50% inhibition index of about 150 under the usual reaction condition as shown in Fig. 1. On the other hand, AMP, adenine or inosine showed no significant effect (Table 4).

The specific inhibition caused by adenosine was observed only when the compound was added prior to the incubation of apoprotein with DBCC. Adenosine seems to compete with the amino group of the deoxyadenosyl moiety of DBCC to combine with the apoprotein.

6. Spectral Changes in the Course of the Enzyme Reaction

Using high concentrations of apoenzyme and DBCC, Abeles and Lee (21) have observed the occurrence of a B_{12r}-like spectrum in the course of the enzymic reaction. An analogous spectral change was also observed in this study. As shown in Fig. 2, the appearance of a B_{12r}-like spectrum having a peak at about 470 mμ

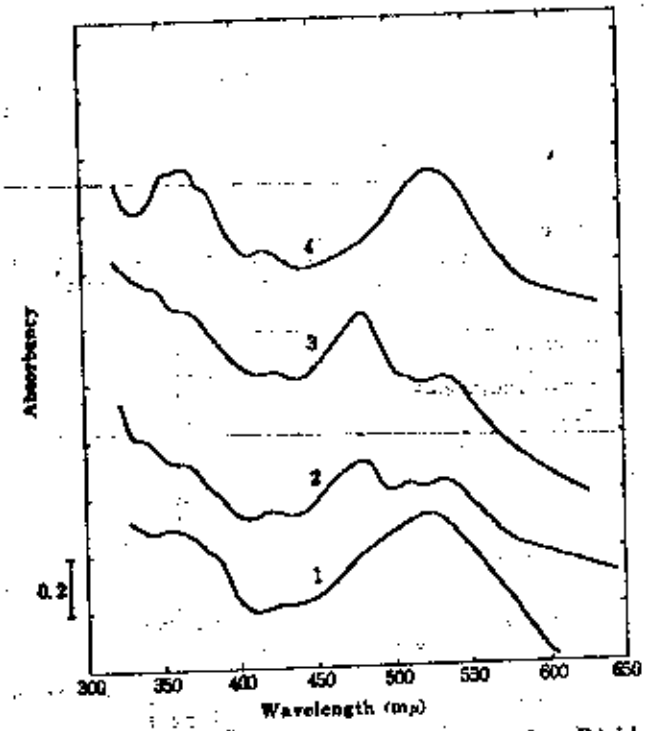

FIG. 2 *Change of the Spectrum of DBCC-dependent Dioldehydrase.* Spectrum 1, holoenzyme only, prior to substrate addition; spectrum 2, 2 minutes after substrate addition; spectrum 3, holoenzyme only, after anaerobic photolysis; spectrum 4, holoenzyme (all substrate consumed at this point). Enzyme 100 units, DBCC 20 μmoles, K-phosphate buffer (pH 8.0) 7 μmoles, volume 0.8 ml. In spectrum 2 and 4, 80 μmoles DL-propanediol was used.

was detected in the enzymic reaction mixture and more obviously in the light-accelerating reaction. This indicates that the enzymic reaction is initiated by the splitting of the cobalt-carbon bond linking 5'-deoxyadenosyl moiety followed by the formation of protein-bound B_{12r}, at least as an intermediary step.

DISCUSSION

Associated with the observations of Lee and Abeles (21) concerning the spectral changes of the dioldehydrase, the results of this study suggest that the effect of the illumination is probably attributed to the stimulation of step (*b*) in the following reaction sequence:

$$E + DBCC \xrightarrow{(a)} E \cdot DBCC \xrightarrow{(b)} E \cdot DBCC^{*}$$

$$\longrightarrow E \cdot DBCC^{*} + S \underset{}{\overset{(c)}{\rightleftharpoons}} E \cdot DBCC^{*} \cdot S \overset{(d)}{\rightleftharpoons}$$

$$E \cdot DBCC^{**} \cdot I \xrightarrow{(e)} E \cdot DBCC^{**} + P$$

In this scheme, E·DBCC represents the apoenzyme-coenzyme complex; E·DBCC*, a complex of the apoenzyme and a modified form of the coenzyme, perhaps having a nucleophilic state of the cobalt atom; I, an intermediate derived from the substrate, S and a precursor of the product P; and E·DBCC** is a presumed state of the holoenzyme after the reaction finishes.

Moreover, an important role of a protein-bound 5'-deoxyadenosyl radical or its further changed form is expected by the ineffectiveness of the irradiation of the incubation mixture of methylcobalamin and the apoenzyme as well as the competitive inhibitory effect of adenosine on the enzymic reaction.

SUMMARY

1. Influences of light-irradiation on the enzymic conversion of propanediol to propionaldehyde catalyzed by 5,6-dimethylbenzimidazolyl cobamide coenzyme-dependent dioldehydrase were examined under the following conditions: (*a*) holoenzyme was irradiated, prior to the incubation with the substrate in the dark, under aerobic and anaerobic conditions, respectively; (*b*) light was irradiated on the mixture of apoenzyme and the coenzyme at 0° where the holoenzyme was hardly formed, then incubated with the substrate in the dark; (*c*) illumination was carried out after the enzymic reaction began to proceed steadily.

2. The irradiation on the holoenzyme under anaerobic conditions stimulated the reaction rate twice that without illumination. On the other hand, the enzyme activity decreased to less than a half under aerobic condition. The accelerating effect of the illumination under anaerobic conditions is considered to be due to the anaerobic cleavage of the cobalt-carbon bond in the enzyme linking a 5'-deoxyadenosyl moiety, which forms a protein-bound B_{12r} and a 5'-deoxyadenosyl radical, even if the protein-bound B_{12r} may be further reduced to the state of B_{12s} before the reaction with the substrate. The ineffectiveness of the aerobic illumination indicates that hydroxocobalamin and adenosine-5'-aldehyde or adenosine-5'-carboxylic acid bound

to the apoprotein cannot react with the substrate. Under the condition *b*, in which the coenzyme was in part transformed to hydroxocobalamin by irradiation, the coenzyme activity decreased to the same extent as that of the aerobic photolysis of the holoenzyme mentioned above. When the enzymic reaction began to proceed steadily (condition *c*), light did not affect the reaction rate.

3. Associated with the appearance of a B_{12r}-like spectrum in the course of the enzymic reaction, the enhancement of the holoenzyme by anaerobic photolysis would be attributed to the stimulation of the cleavage of the cobalt-carbon bond followed by the formation of a protein-bound B_{12r} and 5'-deoxyadenosyl radical, at least as an intermediary step.

4. The ineffectiveness of irradiation on the incubation mixture of the apoenzyme with methylcobalamin, which is known to be a competitive inhibitor in the enzyme system, suggests that the enzymic reaction cannot be mediated by a protein-bound B_{12r} or B_{12s} alone but that protein-bound 5'-deoxyadenosyl part is also necessary for the reaction. The possible participation of 5'-deoxyadenosyl moiety was supported by the competitive inhibition caused by the addition of adenosine.

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The Effect of Vitamin B_{12} on the Synthesis of Phospholipides and Sphingosine in the Developing Chick Embryo[1]

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Introduction

Pernicious anemia is known to be accompanied by a tissue deficiency of vitamin B_{12}, and such patients in relapse were shown to have abnormally low blood phospholipide levels (1–5). This decreased phospholipide concentration was reflected in the three phospholipide components, all of which increased after vitamin B_{12} therapy (5). Ling and Chow (4) presented data on the phospholipide contents of the tissues of vitamin B_{12}-deficient and B_{12}-treated young rats. In the deficient tissue less lipide phosphorus was present, a phenomenon which was corrected by the injection of vitamin B_{12}. More recently, O'Dell and Bruemmer (6) were unable to confirm these observations by P^{32} studies, using new-born rats from vitamin B_{12}-deficient dams.

This paper is a report of studies of phospholipide metabolism in the chick embryo which were conducted in an effort to extend our knowledge of the role of vitamin B_{12} in lipide metabolism.

Experimental

Production and Preparation of Embryos

Twenty-four Single Comb White Leghorn hens were placed in individual cages on raised wire floors. Twelve hens received a vitamin B_{12}-deficient diet and 12 hens received the diet plus 9 μg. B_{12}/lb. diet. The vitamin B_{12}-deficient diet consisted of 50% ground yellow corn, 39.5% soybean oil meal,[4] 3.2% dicalcium phosphate, 1.5%

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[4] Fifty per cent protein, low fiber, solvent-extracted soybean oil meal obtained from Archer-Daniels-Midland Co., Minneapolis, Minn.

ground limestone, 0.5% iodized salt, 0.01% $MnSO_4 \cdot H_2O$, 0.05% DL-methionine, 0.05% choline chloride, 0.088% vitamin A concentrate[5] (10,000 I.U./g.) and 0.073% Delsterol[6] (3000 A.O.A.C. chick units of vitamin D/g.). The following vitamin supplement was added (mg./lb. diet): calcium pantothenate 3.0, riboflavine 2.0, thiamine hydrochloride 1.0, menadione 0.2, and biotin 0.025

The experiment was conducted for a period of 12 weeks. Beginning with the 4th week, the hens were artificially inseminated twice weekly using semen from Single Comb White Leghorn roosters. After 5 weeks on the diet, the hatchability of the eggs from the deficient hens had dropped below 50%. From the 6th through the 12th week, eggs were incubated and living embryos were taken at 12, 15, 18, and 21 days of age. The embryos were dissected from the yolk sac, a small quantity of blood was withdrawn from the exposed heart, and the liver was removed. Five embryos were pooled at 12 days (a total of four composite samples of both normal and vitamin B_{12}-deficient embryos), 3–4 embryos per composite at 15 days (seven composite samples of each), and 2–3 embryos per sample at 18 and 21 days of age (seven composite samples of each at both ages). The composite samples of embryos were homogenized in a Waring blendor and then lyophilized, and the dry weights were recorded. The samples were ground three times in a Wiley mill with Dry Ice using successively finer mesh delivery tubes. The samples were then lyophilized a second time, and stored at −17°C.

Determination of Nitrogen

Approximately 100 mg. of the dried embryonic tissue was digested using the micro-Kjeldahl procedure with 10 mg. $CuSO_4$–K_2SO_4 (3:100 mixture, one selenized Hengar granule, and 2 ml. of conc. H_2SO_4. The digest was made alkaline, and the ammonia was distilled into 0.1 *N* HCl. An aliquot of the distillate was used to determine nitrogen by Nessler's reaction (7).

Isolation, Purification and Determination of Lipide

The phospholipides were extracted from a 2–3 g. sample of the dried embryonic tissue and purified according to the method of McKibbin and Taylor (8), as modified in this laboratory. For purification three extractions with 0.25 *M* $MgCl_2$ were found to be adequate. Lecithin, cephalin, and sphingomyelin were estimated according to the procedure of Hack (9). The digestion procedure and the determination of phosphorus was that of Sumner (10). It was found that incubation at 28°C. for 30 minutes was necessary to give maximum color formation, instead of at room temperature (24°C.) The methods employed for the hydrolysis of sphingolipides and the extraction of sphingosine were those of McKibbin and Taylor (8, 11). Sphingosine was estimated by determining nitrogen by the procedure described.

Results and Discussion

Growth

The average dry weights of the normal and vitamin B_{12}-deficient embryos are shown in Table I. Beginning at 15 days of age until hatching, the embryos from eggs deficient in vitamin B_{12} were smaller. The difference

[5] "Nopcay," obtained from the Nopco Chemical Co., Harrison, N. J.

[6] Activated animal sterol obtained from E. I. du Pont de Nemours & Co., Wilmington, Del.

TABLE I

Effect of Vitamin B_{12} Deficiency on the Dry Weight and Total Nitrogen Content during Embryonic Development of the Chick[a]

Age of embryo (days)	Composite samples n[b]	Av. dry weight embryo g	Av. total nitrogen embryo g
	Normal		
12	4 (20)[b]	0.39 ± 0.02[c]	—
15	7 (21)	1.84 ± 0.21	0.10 ± 0.03
18	7 (20)	4.26 ± 0.28	0.42 ± 0.02
21 (hatching)	7 (19)	6.73 ± 0.25	0.64 ± 0.03
	Vitamin B_{12}-deficient		
12	4 (20)	0.39 ± 0.03	—
15	7 (22)	1.65 ± 0.28	0.18 ± 0.03
18	7 (20)	3.55[d] ± 0.36	0.36[d] ± 0.03
21	7 (19)	5.80[d] ± 0.73	0.55[d] ± 0.09

[a] The liver and 0.02–0.1 ml. of blood were removed from each embryo.

[b] Number in parentheses represents total number of embryos.

[c] Standard deviation.

[d] The difference between the normal value and the corresponding value for the vitamin B_{12}-deficient embryo is statistically significant at the 5% level.

[e] This denotes a statistically significant difference at the 1% level.

in dry weight between the normal and vitamin B_{12}-deficient embryos was statistically significant at 18 and 21 days of age.

Although the average B_{12} content of the B_{12}-deficient eggs before incubation was markedly less than that of the normal eggs (3.8 mμg. vs. 57.7 mμg. g. dry weight), there was no decrease in the dry weight of the egg due to a B_{12} deficiency: 14.2 g., normal, and 14.0 g., B_{12}-deficient. It is possible that the composition of these eggs was different. However, we have found that the injection of vitamin B_{12} directly into the yolk of these fertile eggs at 3 days of age increased the hatchability markedly. Similar findings have been recorded by others (12, 13).

The nitrogen contents of these embryos are also presented in Table I. The average nitrogen content of embryos from eggs deficient in vitamin B_{12}, as compared to the normal, was significantly lower at 18 and 21 days. A similar trend was noted at 15 days, although the difference was not significant.

Phospholipide

The amount of total phospholipide, lecithin, cephalin, and sphingomyelin found in the control and deficient embryos are presented in Table II. The most rapid synthesis of total phospholipide occurred between 12 and 18 days of age. This was reflected in each of the components except sphingo-

TABLE II

Phospholipide Changes during Embryonic Development of the Chick[a]

Age of embryo days	Av. phospholipide phosphorus/embryo mg.	Av. lecithin choline/embryo mg.	Av. cephalin phosphorus/embryo mg.	Av. sphingomyelin phosphorus/embryo mg.
		Normal		
12	1.29[b] ± 0.05[c]	2.53 ± 0.24	0.44 ± 0.05	0.21 ± 0.06
15	3.99 ± 0.36	7.55 ± 0.80	1.37 ± 0.13	0.66 ± 0.18
18	6.60 ± 0.70	11.35 ± 1.53	2.12 ± 0.20	1.04 ± 0.19
21	8.90 ± 0.66	15.06 ± 1.09	2.25 ± 0.27	2.32 ± 0.37
		Vitamin B_{12}-deficient		
12	1.28 ± 0.14	2.52 ± 0.19	0.40 ± 0.05	0.18 ± 0.08
15	3.80 ± 0.35	7.70 ± 0.76	1.11 ± 0.22	0.13 ± 0.17
18	6.01 ± 0.70	11.13 ± 1.32	1.71[d] ± 0.27	1.45 ± 0.41
21	7.44[e] ± 0.81	13.95[f] ± 1.42	1.79[f] ± 0.52	1.79[f] ± 0.21

[a] The liver and 0.02–0.1 ml. of blood were removed from each embryo.

[b] All values are averages of the number of composite samples shown in Table I.

[c] Standard deviation.

[d] The difference between the normal value and the corresponding value for the vitamin B_{12}-deficient embryo is statistically significant at the 5% level.

[e] This denotes a statistically significant difference at the 1% level.

myelin, which had its greatest increase between 15 and 18 days of age. These results are in accord with those of Tsuji, Brin, and Williams (14).

Total phospholipide synthesis was decreased in the vitamin B_{12}-deficient embryo as compared to the control, corroborating the findings of Ling and Chow (4). This trend was first observed in the deficient embryo at 15 days of embryonic age, and at the time of hatching (21 days) the difference was statistically significant at the 1% level. The three phospholipide components, lecithin, cephalin, and sphingomyelin, were also lower in the B_{12}-deficient embryo. At 18 days of embryonic age, cephalin was the only fraction which was significantly lower in the B_{12}-deficient embryo. Whereas, at 21 days of age, all fractions were significantly decreased in the deficient embryo.

In a vitamin B_{12} deficiency, growth retardation occurs in many animals. Since this was the case with these embryos, it is possible that the decreased synthesis of the phospholipides may have resulted either from an indirect effect of B_{12} on growth through its action in protein or nucleic acid metabolism, or from a more direct effect of B_{12} on phospholipide synthesis. These data were examined further in order to evaluate these possibilities.

In Figure 1 the relationship of the change in total nitrogen was plotted as a function of the dry weight of the embryo. Both the ordinate and abscissa are logarithmic in scale. The synthesis of protein was not apparently limiting in the B_{12}-deficient embryo for the ratio of total nitrogen

to dry weight of the embryo was equivalent to that observed for the normal embryo during the period studied. Similar observations have been made by others (15–17) using the rat.

The phospholipide data were examined in a similar manner. In Fig. 2,

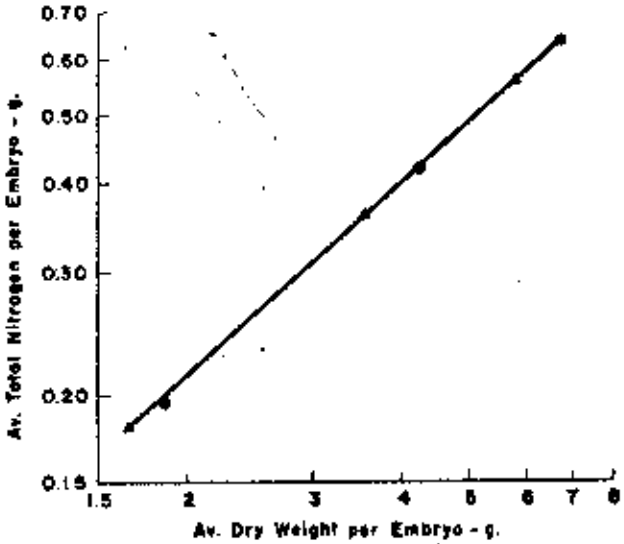

FIG. 1. Relationship of total nitrogen increase to growth of chick embryo. ●—●$ + B_{12}$, ▲—▲$ - B_{12}$. The grouping of points from left to right represent values for 15-, 18-, and 21-day-old embryos.

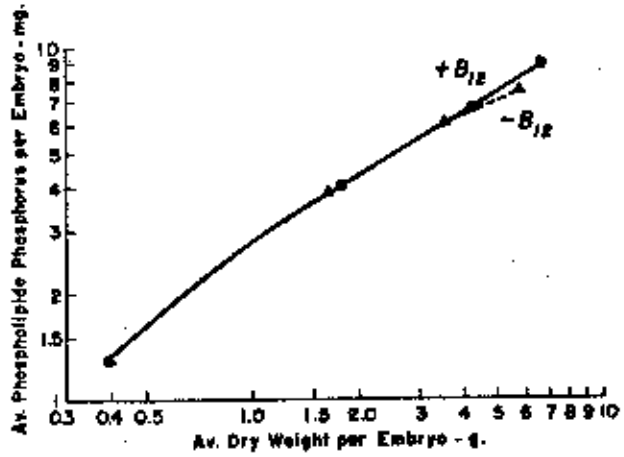

FIG. 2. Relationship of phospholipide synthesis to growth of chick embryo. The grouping of points from let to right represent values for 12-, 15-, 18-, and 21-day-old embryos.

total phospholipide was plotted as a function of growth of the embryo expressed as dry weight. It is evident that growth and phospholipide synthesis in the normal embryo proceeded at a constant rate from 15 days to hatching, as shown by the straight line. For the vitamin B_{12}-deficient embryo at 12, 15, and 18 days of age, the same ratio of lipide phosphorus to dry weight was maintained as noted for the normal embryo. However, at 21 days the phospholipide synthesis was impaired to a greater extent

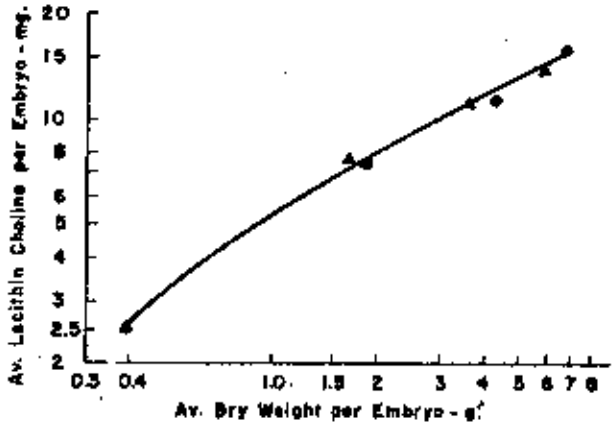

FIG. 3. Relationship of lecithin synthesis to growth of chick embryo. ●---● + B_{12}, ▲——▲ − B_{12}. The grouping of points from left to right represent values for 12-, 15-, 18-, and 21-day-old embryos.

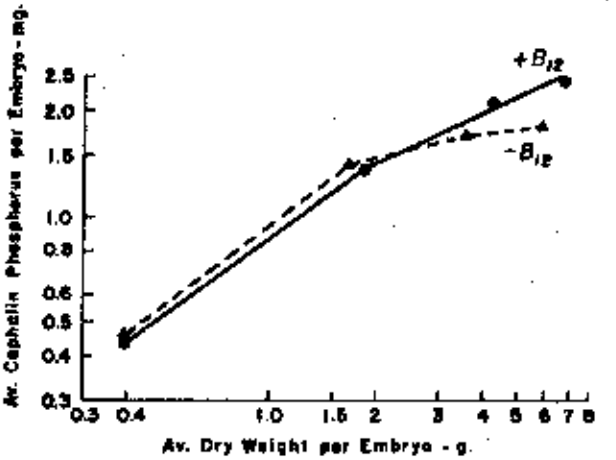

FIG. 4. Relationship of cephalin synthesis to growth of chick embryo. The grouping of points from left to right represent values for 12-, 15-, 18-, and 21-day-old embryos.

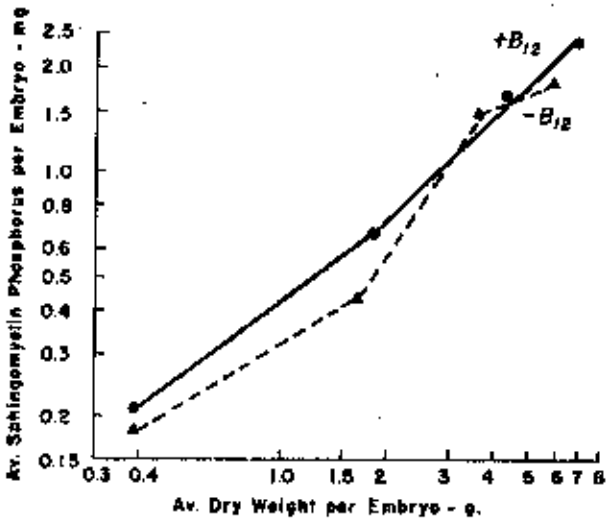

FIG. 5. Relationship of sphingomyelin synthesis to growth of chick embryo. The grouping of points from left to right represent values for 12-, 15-, 18-, and 21-day-old embryos.

than was growth, since the value fell below the normal curve. The two-dimensional logarithmic plot has minimized this difference; however, it is highly significant. If phospholipide level per embryo is plotted as a function of the nitrogen content, the same curves are obtained. Therefore, it is apparent a vitamin B_{12} deficiency had affected phospholipide synthesis of the embryo to a greater degree than it had growth or protein synthesis.

In Figs. 3–5 are plotted the amounts of the individual phospholipides synthesized as a function of the growth of the embryo. The ratio of lecithin to dry weight of the embryo remained the same for both types of embryos at all ages. However, the synthesis of cephalin and sphingomyelin at 21 days and cephalin at 18 days was retarded to a greater extent by a B_{12} deficiency than was the growth of the embryo. These data suggest that a vitamin B_{12} deficiency interferes with cephalin and sphingomyelin synthesis to a greater extent than with lecithin synthesis. These results might be expected since the rate of lecithin synthesis is greater than that of cephalin and sphingomyelin in the early development of the chick embryo (Table II) (14). Thus, the B_{12} present in the deficient embryo was sufficient to maintain the ratio of lecithin to dry weight during the entire incubation period, whereas, the B_{12} level was not adequate to maintain the ratio of cephalin or sphingomyelin to dry weight during the later stages of embryonic development.

TABLE UI

Effect of Vitamin B_{12} Deficiency on Sphingosine Synthesis during Embryonic Development of the Chick

Age of embryo	Average wt. sphingosine nitrogen/embryo	
	Normal	Vitamin B_{12}-deficient
days	mg.	mg.
15	418[b] ± 87[c]	376 ± 43
18	836 ± 72	630[d] ± 89
21	1402 ± 351	1282 ± 220

[a] The liver and 0.02–0.1 ml. of blood were removed from each embryo.
[b] All values are averages of the number of composite samples shown in Table I.
[c] Standard deviation.
[d] The difference between the normal value and the corresponding value for the vitamin B_{12}-deficient embryo is statistically significant at the 1% level.

Sphingosine

The decreased amount of sphingomyelin in the B_{12}-deficient tissue could be due to an inadequate synthesis of sphingosine, a precursor of sphingomyelin. The effect of vitamin B_{12} deficiency on sphingosine synthesis during embryonic development is shown in Table III. At 18 days of age the decrease in sphingosine observed for the deficient embryo was significant at the 1% level. Although there was less sphingosine in the deficient embryo at 15 and 21 days of age, the differences were not significant. When the relationship of sphingosine synthesis to growth as affected by a vitamin B_{12} deficiency was plotted on two-dimensional logarithmic paper, it was found that the decrease in sphingosine synthesis was directly proportional to growth retardation. Therefore sphingosine synthesis was apparently not a limiting factor for sphingomyelin formation.

Acknowledgement

The authors are indebted to the Poultry Department for supplying the chick embryos used in this work.

Summary

Total phospholipide and the three fractions, lecithin, cephalin, and sphingomyelin, were decreased in the vitamin B_{12}-deficient chick embryo. Although growth of the deficient embryo as measured by dry weight or total nitrogen was significantly retarded at 18 and 21 days of age, the total phospholipide content of the embryo at 21 days was reduced to an even greater extent. The three phospholipide fractions were not decreased proportionately in a B_{12} deficiency. Lecithin decrease was correlated with

growth retardation, whereas both cephalin and sphingomyelin were decreased to a greater extent than was growth.

A decrease in the synthesis of sphingosine was noted in the B_{12}-deficient embryo. However, the decreased synthesis was directly proportional to growth retardation, indicating that sphingosine synthesis was not a limiting factor for sphingomyelin formation.

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SENSITIVITY TO VITAMIN B_{12} CONCENTRATE

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It has been suggested that vitamin B_{12} is satisfactory therapy for patients who are sensitive to liver.[1] This report concerns a patient extremely sensitive to liver extract who also reacted to vitamin B_{12} concentrate but showed no evidence of sensitivity to crystalline vitamin B_{12}.

REPORT OF CASE

The patient was known to have had pernicious anemia for five years and was first treated with injections of purified liver extract. He improved and voluntarily discontinued therapy after one year's treatment. One and one-half years later treatment was resumed with purified beef liver extract. The patient improved but later experienced reactions to injectable liver extract and was started on oral therapy with liver-stomach concentrate and iron. Therapy was adequate for some time, but in August 1949 the man had neurologic and hematologic relapse. It was learned that he had been taking folic acid 7.5 mg. daily in addition to liver-stomach concentrate with iron for about one year. When injectable liver therapy was resumed he experienced severe and alarming reactions after each dose, but in spite of these reactions he improved clinically. He received several large doses of thiamine hydrochloride during this relapse.

The patient was found to be extremely sensitive to purified liver extract with added thiamine hydrochloride (3 mg. per cubic centimeter) for parenteral use (reticulogen®) and was admitted to the ward of the Lilly Laboratory for Clinical Research. Although desensitization was accomplished with difficulty, it was possible after four days to administer 1 cc. of the drug without reaction. The patient was discharged from

From the Lilly Laboratory for Clinical Research, Indianapolis General Hospital.

1. Beck, F.; Denny-Brown, D.; Finland, M., and Castle, W. B.: Effectiveness of Vitamin B_{12} in Combined System Disease, New England J. Med. 239:328-330 (Aug. 26) 1948.

the hospital to receive 0.5 cc. of the drug intramuscularly twice weekly. Within two months he again had severe reactions to the injections.

Treatment was changed to vitamin B_{12} concentrate made from streptomycin broth, the equivalent of 5 micrograms every five days. After the sixth dose the patient complained of a slight burning of his throat and itching and swelling at the site of injection. He received five or six more injections and experienced similar discomfort each time. The medication was changed to a vitamin B_{12} concentrate made from liver (experimental no. 1310), and 5 micrograms was administered every five days for six months without untoward reaction. During this time the blood became normal, and there was decided improvement in neurologic symptoms. The patient returned to work. Since the supply of vitamin B_{12} concentrate made from liver was exhausted, a vitamin B_{12} concentrate prepared from streptomycin broth (experimental no. 1336) was administered. About five to ten minutes after the patient received the eleventh injection of 5 micrograms of the vitamin B_{12} concentrate prepared from streptomycin broth peripheral circulatory collapse developed. The patient was perspiring profusely. The blood pressure was approximately 40 systolic and zero diastolic; the pulse was rapid and weak. He was given an infusion of isotonic sodium chloride solution, injections of epinephrine and antihistaminic drugs by the oral route. He recovered after approximately three hours. Therapy was then changed to crystalline vitamin B_{12} made from streptomycin broth, and the patient has shown no evidence of sensitivity.

Intradermal skin tests were performed with 1:100,000 dilutions of purified liver extract, 15 injectable units per cubic centimeter with purified liver extract with added thiamine hydrochloride for parenteral use and with a 1:1,000 dilution of vitamin B_{12} concentrate from liver (experimental no. 1310). Wheals with pseudopodia and erythema developed at the sites of injection. The reaction to a 1:1,000 solution of vitamin B_{12} concentrate from streptomycin broth (experimental no. 1336) consisted of an area of erythema approximately 1.5 cm. in diameter. Tests were also made with a 1:1,000 dilution of thiamine hydrochloride, with crystalline vitamin B_{12} and with streptomycin. The site of injection of the thiamine hydrochloride showed an area of slight erythema, approximately 1 cm. in diameter. There was no reaction at the site of injection of crystalline vitamin B_{12} and streptomycin.

SUMMARY

A patient with pernicious anemia who is highly sensitive to purified liver extracts made from beef liver and pork liver has experienced reactions to vitamin B_{12} concentrate made from streptomycin broth and showed evidence of acquired sensitivity to vitamin B_{12} concentrate made from liver, as manifested by the intradermal skin test. As yet there is no evidence of sensitivity to crystalline vitamin B_{12}.

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Vitamin B_{12}
intramuscular administration
Vitamin C
oral supplementation
saturation levels

metabolic interrelationship
protein origin
vegetarian diets
– cereals, millets

Effect of Vitamin B_{12} Administration on Ascorbic Acid Levels in Plasma and Leucocytes in Human Subjects

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Summary: The effect of administration of vitamin B_{12} on ascorbic acid levels in plasma and leucocytes was investigated in 9 apparently normal adult subjects who were maintained on vegetarian diets and had low levels of plasma vitamin B_{12}. Administration of vitamin B_{12} had no influence on either plasma or leucocytes after the subjects were saturated with ascorbic acid. Administration of ascorbic acid along with vitamin B_{12} raised the concentration of ascorbic acid in plasma and leucocytes to the same level as those obtained with ascorbic acid supplementation alone.

There have been several reports indicating the existence of a metabolic interrelationship between vitamin B_{12} and ascorbic acid in man [1, 5, 9]. It was observed that in some vitamin B_{12} deficient subjects plasma ascorbic acid levels were low and that they could not be raised to normal levels even with large dose of ascorbic acid unless vitamin B_{12} wasalso administered [1, 5]. We had reported earlier that the mean levels of ascorbic acid both in plasma and in leucocytes of apparently normal Indian subjects, who were fully saturated with the vitamin, were found to be much lower than those reported from other countries [6]. Most of the subjects investigated in that study were on vegetarian diets, based predominantly on cereals and millets. Though none of the subjects had evidence of abnormal haemopoiesis, plasma levels of vitamin B_{12} were in the "deficient" range in many of them. It was considered important to determine whether suboptimal vitamin B_{12} nutritional status had in any way influenced the "saturated" levels of ascorbic acid in plasma and leucocytes.

Materials and methods

Nine apparently normal adult volunteers were investigated. They were maintained on a diet which provided about 2400 kt and 60–70 g protein daily. All the protein was of vegetable origin. Levels of vitamin B_{12} in plasma and concentration of ascorbic acid in plasma and leucocytes were determined prior to the start of the study. To four of the nine subjects (group I), 500 mg ascorbic acid was supplemented daily, orally until the leucocytes were saturated as indicated by a constant value on two successive determinations done at intervals of a week. Levels of vitamin B_{12} in plasma were simultaneously determined. They were then given 50 µg cyanocobalamin intramuscularly on alternate days for about 20 days. The levels of plasma vitamin B_{12}, ascorbic acid in plasma and leucocytes were determined at the end of vitamin B_{12} administration. Five other subjects (group II) received 500 mg ascorbic acid/day orally and 50 µg cyanocobalamin intramuscularily on alternate day from the beginning for a period of 20 days. A total of 500 µg cyanocobalamin was thus administered.

During the study, it was observed that subjects receiving the ascorbic acid supplements tended to show a fall in their plasma vitamin B_{12} levels. To determine whether this was due to an increased demand of vitamin B_{12} for utilization of the vegetable protein provided in the diet during the study or due to ingestion of large amounts of ascorbic acid *per se*, three normal subjects (group III), in whom the initial levels of plasma vitamin B_{12} were determined, were maintained on diets identical with those employed for subjects in groups I and II, for a period of 15 days. They received no supplements of ascorbic acid.

All samples of blood for analysis were obtained under basal conditions. Ascorbic acid concentration in plasma was measured by the method of Roe and Kuether [7]. Leucocyte ascorbic acid was estimated by the method described by Dodson and Bowrai [2]. Vitamin B_{12} was extracted from plasma by the method of Gregory [3] and activity of the vitamin was assayed by using *Euglena gracilis* (Z strain) as the test organism according to the method of Hutner and Bach [4].

Results

It was observed that administration of vitamin B_{12} had no influence on ascorbic acid levels either in plasma or leucocytes after "saturation" levels had been reached. Following administration of ascorbic acid along with vitamin B_{12}, the levels of ascorbic acid in plasma and leucocytes were found to be 1.41 ± 0.13 mg/100 ml and 16.4 ± 1.74 µg/10^8 cells respectively (Fig. 2). These values were similar to those obtained with supplementation of ascorbic acid alone, the levels being 1.3 ± 0.15 mg/100 ml in plasma and 14.9 ± 0.83 µg/10^8 in leucocytes (Fig. 1).

In subjects receiving ascorbic acid supplements alone, the mean level of plasma vitamin B_{12} before and after supplementation of ascorbic acid were 91.0 ± 29.56 and 87.6 ± 10.38 pg/ml respectively. This fall in the concentration of vitamin B_{12} in plasma was statistically not significant. However, in three of four subjects, there was a fall of 10.0, 20.2 and 83.2 pg/ml. Following on administration of vitamin B_{12} to these subjects, the levels increased significantly (mean = 280.6 ± 38.46 pg/ml); $p < 0.001$ – fig. 1). In subjects receiving ascorbic acid along with vitamin B_{12} from the start of the experiment the mean initial levels of plasma

vitamin B_{12} was 105.3 ± 26.09 pg/ml which increased significantly to a level of 412.5 ± 56.55 pg/ml after the administration of the vitamin (Fig. 2). The initial level of plasma vitamin B_{12} was below 100 pg/ml in 7 of the 9 subjects.

In subjects who were maintained on identical diets, but did not receive ascorbic acid supplements, the initial levels of plasma vitamin B_{12} were 98.4, 80.2 and 87.5 pg/ml. At the end of the experimental period, the levels of plasma vitamin B_{12} had fallen to 75.0, 81.5 and 72.0 pg/ml respectively.

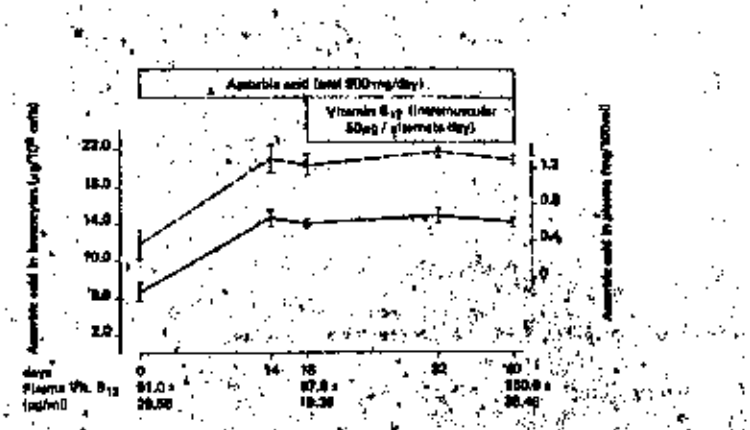

Fig. 1: Effect of vitamin B_{12} administration on the levels of ascorbic acid in plasma and leucocytes in 4 subjects (group I). Values for ascorbic acid and vitamin B_{12} are expressed as Mean ± S.E.

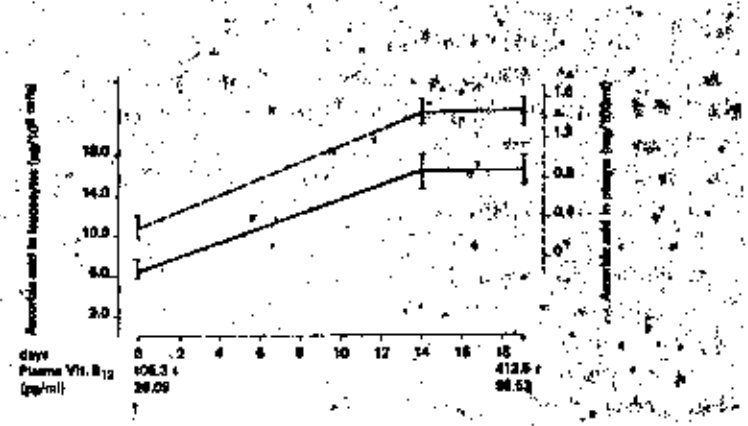

Fig. 2: Effect of simultaneous administration of ascorbic acid and vitamin B_{12} on the levels of ascorbic acid in plasma and leucocytes in 5 subjects (group II). Values for ascorbic acid and vitamin B_{12} are expressed as Mean ± S. E. (intramuscular 50 µg/every alternate day) Administration of ascorbic acid (oral 500 mg/day) and vitamin B_{12}

Discussion

In these experiments, two approaches were tried to ascertain whether the low levels of ascorbic acid observed even after saturation, were due to concomitant deficiency of vitamin B_{12} – (a) to see if the administration of vitamin B_{12} to subjects already saturated with respect to ascorbic acid would further raise the levels of the vitamin in leucocytes; (b) to see whether the simultaneous administration of ascorbic acid and vitamin B_{12} from the beginning would result in saturated levels higher than those observed with ascorbic acid supplementation alone.

The results of these studies indicated no relationship between the two vitamins – an observation contrary to that reported earlier [1, 5, 9]. The data reported by Kapp and Bhoosry [5] pertain to a single subject who had multiple deficiency signs and who was treated with diets providing large amounts of animal protein. The changes observed in ascorbic acid levels in plasma following the feeding of animal protein diets cannot be attributed to vitamin B_{12} alone. The results of the present study show that the low levels of ascorbic acid in the subjects investigated here cannot be explained on the basis of their vitamin B_{12} status. This is in line with our earlier observations that subjects belonging to the well-to-do group had normal levels of vitamin B_{12} and yet had relatively low levels of ascorbic acid in leucocytes after full saturation, similar to those observed in the subjects belonging to the low income group [6]. Urinary excretion of methyl melonic acid was not studied in the subjects investigated here and hence it is difficult to judge their precise status with regard to vitamin B_{12}. However, accepting plasma levels of the vitamin below 100 pg/ml as deficient, the vitamin B_{12} status of the subjects may be considered to be suboptimal in the subjects studied here.

The fall in plasma vitamin B_{12} levels observed in subjects who received ascorbic acid supplements could be due to (a) an effect of ascorbic acid supplementation *per se* or (b) an increased demand for available B_{12} for optimal utilisation of the vegetable protein diet.

The fall in vitamin B_{12} levels in the three normal subjects who were maintained on vegetable diets for 18 days and received no additional supplements of ascorbic acid supports the view that this fall was due to the diet *per se* and not related to the ascorbic acid supplementation. These results are also in line with the observations made earlier by Sattanarayana [8] that diets providing high amounts of vegetable protein increase vitamin B_{12} requirements.

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